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Dairy Farm Age and Resistance to Antimicrobial Agents in *Escherichia coli* Isolated from Dairy Topsoil

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Abstract

Antimicrobial agent usage is common in animal agriculture for therapeutic and prophylactic purposes. Selective pressure exerted by these antimicrobials on soil bacteria could result in the selection of strains that are resistant due to chromosomal or plasmid-derived genetic components. Multiple antimicrobial resistances in *Escherichia coli* and the direct relationship between antimicrobial agent use over time has been extensively studied, yet the relationship between the age of an animal agriculture environment such as a dairy farm and antibiotic resistance remains unclear. Therefore, we tested the hypothesis that antimicrobial resistance profiles of *E. coli* isolated from dairy farm topsoil correlate with dairy farm age. *E. coli* isolated from eleven dairy farms of varying ages within Roosevelt County, NM were used for MIC determinations to chloramphenicol, nalidixic acid, penicillin, tetracycline, ampicillin, amoxicillin/clavulanic acid, gentamicin, trimethoprim/sulfamethoxazole, cefotaxime and ciprofloxacin. The minimum inhibitory concentration values of four antibiotics ranged between 0.75 - >256 µg/ml, 1 - >256 µg/ml, 12 - >256 µg/ml, and 0.75 - >256 µg/ml for chloramphenicol, nalidixic acid, penicillin and tetracycline, respectively. The study did not show a direct relationship between antibiotic resistance and the age of dairy farms.

Keywords

antimicrobial resistance; MIC; dairy soil; dairy age; *marRAB*

Introduction

The widespread use of antimicrobial agents in animal agriculture has raised public concerns regarding zoonotic transmission of antibiotic-resistant bacteria [27]. A recent study reviewed antimicrobial usage in Pennsylvania dairy herds and found that 24 antimicrobial agents were used extensively on farms to promote growth and prophylaxis in dairy cattle [25]. These antimicrobial agents were frequently used to treat pneumonia, enteritis, and foot infections. Furthermore, tetracycline-resistant *Escherichia coli* organisms in the dairy cattle were isolated, and it was determined that the genetic-determinants conferring this resistance were located on the bacterial chromosomes [25]. This study indicates that commensal enteric *E. coli* from healthy cattle may comprise a huge reservoir of antimicrobial-resistant pathogens that could potentially contaminate the food chain.

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In the presence of antimicrobials, microbes evolve highly effective mechanisms to cope with the negative selection pressures that antibiotics exert [32]. Antimicrobial-resistance in microorganisms may be conferred by enhanced bacterial physiological processes such as membrane transport pumps, acquisition of extrachromosomal elements, or spontaneous mutations in the bacterial genome, and may potentially be a problem with serious implications on public health [28]. Antimicrobial resistance genes can be disseminated via direct contact with tainted food, animal feces, and contaminated ground [2,5,15,17,30]. Alternatively, soil may also serve as a potential reservoir for antimicrobial-resistance dissemination [8]. Despite their efficacy, many antibiotics are poorly absorbed by the treated animal, and as much as 25–75% of antibiotics administered could be excreted in the feces and persist in soil [5,15]. Consequently, concerns have been raised about the emergence of low-level and multiple antibiotic-resistant soil bacteria due to the presence of sub-therapeutic antibiotic levels in soil [5].

In the presence of antibiotics, it is unknown to what extent chromosomally encoded multiple antibiotic resistance genes play in mediating multidrug resistance in agriculture. The *marRAB* operon is a 1.1 kb multiple antibiotic resistance operon that controls the expression of chromosomal genes involved in mediating multidrug resistance in members of the Enterobacteriaceae. Specifically, this operon is induced by a variety of compounds such as salicylic acid, antimicrobials such as tetracycline and chloramphenicol, with the former being used in agriculture [7]. The *marRAB* operon was found to confer reduced susceptibilities to the antimicrobial agents trimethoprim, gatifloxacin, cefotaxime and tetracycline in *E. coli* species recovered from pasteurized whole milk [22].

Our laboratory has demonstrated that dairy farm topsoil from Roosevelt County, New Mexico harbors enteric bacteria that are multiple antimicrobial-resistant via the *marRAB* operon [4]. Although antibiotic-resistant bacteria are a consequence of evolutionary adaptations, the increasing use and possible misuse of antibiotics creates a situation in need of efficient monitoring and surveillance of resistance trends [27].

Although it is known that *E. coli* isolates may be antimicrobial-resistant, it is not known whether there is a direct relationship between antibiotic resistance mediated by the *mar* operon and the age of the animal environment. Numerous studies have shown a highly significant positive correlation between the introduction of antimicrobials into agriculture and the development of bacterial antimicrobial resistance [27]. Higher prevalence of multidrug-resistant *E. coli* was found in animals that were treated with antimicrobials compared to those from organic farms in which no antimicrobials were used [24]. We therefore hypothesized that there may be a direct correlation between dairy farm age and the time in which antimicrobial agents are used. Here, we examine the effects of the age of dairy farms in Roosevelt County, NM on the antibiotic resistance in *E. coli*.

Materials and Methods

Sample Collection

The topsoil samples were obtained from 11 randomly selected dairy farms of varying ages (0.5, 1, 5, 7, 12, 10, 20, and 24 years) in Roosevelt County, New Mexico. A total of nine samples were obtained from each dairy farm, three samples from randomly selected corrals. Approximately five grams of topsoil were obtained in each sample, transferred into 20 mL of LB broth, shaken, and placed on ice for 20 minutes.

Bacterial isolation, Storage, and Identification

Soil samples were centrifuged at 4°C and 13,000 × *g* for 30 sec. Twenty microliters of supernatant were plated onto MacConkey (BD Diagnostics, Sparks, MD) agar plates

containing 1% lactose, and incubated at 37°C for 24 h. Individual colonies were picked and grown separately in Luria-Bertani (LB) broth (BD Difco, Sparks, MD) at 37°C for 24 h. Isolates were stored in 30% glycerol at -70°C. MacConkey agar plates supplemented with 1% lactose were inoculated using either frozen bacterial stocks or freshly grown cultures. Isolates were incubated at 37°C for 24 hours and identified using the BBL Crystal Identification System as specified by the manufacturer (BD Diagnostics, Sparks, MD). In addition, 22 *E. coli* isolates from a previous study conducted in this laboratory were included in the present study (4).

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) for *E. coli* isolates to a panel of 6 antimicrobial agents were determined using E-test strips (AB Biodisk, Piscataway, NJ) as specified by the manufacturer and according to the National Committee for Clinical Laboratory Standards (CLSI) [6]. Briefly, the isolates were grown overnight on MacConkey (Becton Dickinson, Sparks, MD) agar plates, and individual colonies were resuspended to 0.5 McFarland in 0.85% NaCl and re-plated over the surface of Mueller Hinton (Becton Dickinson, Sparks, MD) agar plates (150 mm). Subsequently, antimicrobial E-test strips (ampicillin, gentamicin, ciprofloxacin, cefotaxime, trimethoprim/sulfamethoxazole, and amoxicillin/clavulanic acid) were applied to each plate and incubated at 37°C for 18 hours. Vancomycin was used as a negative control.

A second panel of *marRAB*-associated antibiotics (tetracycline, penicillin G, chloramphenicol, and nalidixic acid) was used to determine the level of isolate resistance by the gradient plate method as previously described [29]. Isolates were scored for susceptibilities and resistances according to CLSI standards [6]. Isolates were grown to mid-log phase in LB broth (Becton Dickinson, Sparks, MD) at 30°C and streaked with sterile cotton swabs onto Luria agar antibiotic gradient plates. The plates were incubated for 40 h at 30°C. MICs were determined by interpolation of the relative lengths of growth, assuming a linear gradient across the plate. The gradient plate method was used for this panel of antibiotics as nalidixic acid and penicillin G E-test strips were not available.

Analysis of the *marRAB* Locus Phenotype

Induction of the *marRAB* operon was assessed by using slight modifications, described below, to the previously described methods (12, 14). Bacteria were grown to mid-log phase in Mueller Hinton broth at 30°C and streaked with sterile cotton swabs onto Mueller Hinton agar gradient plates supplemented with 5mM salicylate. The plates were incubated for 40 hours at 30°C. The MICs were determined by interpolation of the relative lengths of growth, assuming a linear gradient across the plate.

PCR primers and Reaction Conditions

Polymerase chain reaction was performed using primers and protocols described previously to detect *ial*, *estA* [33], *eltB*, *eaeA* [10], *aggR* [26], *invE*, *stx₁* and *stx₂* [16] and *bfpA* [31], virulence genes commonly associated with EPEC, EAEC, EIEC, ETEC and STEC pathogenic serotypes. All *E. coli* isolates were grown in 3 mL of LB broth for 18 h and centrifuged at 10,000 g for 10 minutes. The resultant pellet was resuspended in 300 µl sterile distilled water and boiled for 10 min to lyse the cells. Each lysate (5 µl) was amplified using gene specific primers (Table 1) in a 25 µl reaction mixture containing 2.5 µl of 10× PCR buffer, 50 mM MgSO₄, 0.5 µl of a mixture of 200 µM concentrations of each of the four dNTPs, and 1U *Taq* polymerase (Invitrogen, USA). The products were separated on 2% agarose gel stained with ethidium bromide (0.5 µg/ml). PCR results were compared to positive controls consisting of *E. coli* pathotypes EPEC E2348/69, ETEC H10407, EIEC

EDL1284 and STEC EDL933, while the DNA from *E. coli* K12 served as the negative control for PCR reactions.

Statistical Analyses

To test the effects of dairy farm age on *E. coli* resistance to the specified antimicrobial agents, E-test MIC data in triplicate were tested for normality using the Kolmogorov-Smirnov normality test which revealed that MIC data for each antimicrobial were not normal ($P < 0.001$). Therefore a non-parametric spearman rank correlation analysis was used as described [34]. *E. coli* MICs represented as $>256 \mu\text{g/mL}$ on the E-test strip were not utilized in the statistical analysis due to the inability to determine the exact MIC values. A one-tailed test was used because the priori hypothesis was that antibiotic resistance to the antibiotics would increase in older dairy farms. Results were considered significant at $P < 0.05$.

Results

No Correlation between Dairy Farm Age and *E. coli* Antimicrobial Susceptibility

Five of 33 *E. coli* isolates demonstrated high-level resistance to tetracycline, $>256 \mu\text{g/mL}$, while the remaining *E. coli* isolates demonstrated varying levels of resistance from 0.96–74.6 $\mu\text{g/mL}$ (Figure 1A). Most *E. coli* strains demonstrated low-level resistance to chloramphenicol, nalidixic acid, penicillin, and tetracycline. As shown in Figure 1B, one of 38 *E. coli* strains showed the highest resistance to chloramphenicol, 54 $\mu\text{g/mL}$. Slightly higher resistance was observed to nalidixic acid, 1.5–12.5 $\mu\text{g/mL}$, with the exception of three of 37 *E. coli* isolates that conferred high-level resistances of $>256 \mu\text{g/mL}$ (Figure 1C). The highest resistance, however, was noted to penicillin, with MICs of 12.0 - $>256 \mu\text{g/mL}$ (Figure 1D).

For the panel of non-*marRAB* associated antimicrobial agents, including ampicillin, ciprofloxacin, cefotaxime, gentamicin, trimethoprim/sulfamethoxazole and amoxicillin/clavulanic acid, no resistant organisms were found, as compared to the established breakpoints specified by the National Committee for Clinical Laboratory Standards (Fig. 2A–D and 3 A–B). The mean MIC values of *E. coli* isolates corresponding to different dairy ages are shown in Table 1. Positive but not significant correlations existed between dairy farm age and *E. coli* susceptibility to ampicillin ($r_s = 0.130$, $n = 43$, $P > 0.05$), trimethoprim/sulfamethoxazole ($r_s = 0.352$, $n = 23$, $p > 0.05$), ciprofloxacin ($r_s = 0.0740$, $n = 41$, $P > 0.05$), and gentamicin ($r_s = 0.204$, $n = 42$, $p > 0.05$). Negative but not significant correlations were found between dairy farm age and *E. coli* susceptibility to ciprofloxacin/cefotaxime ($r_s = -0.0211$, $n = 42$, $P > 0.05$), and amoxicillin/clavulanic acid ($r_s = -0.115$, $n = 43$, $P > 0.05$). According to our study, we found that there is no significant correlation between dairy farm age and the susceptibilities of *E. coli* isolates to the antimicrobials ampicillin, trimethoprim/sulfamethoxazole, ciprofloxacin, cefotaxime, amoxicillin/clavulanic acid, and gentamicin.

marRAB Locus Activity is Up-regulated in Presence of Salicylate

Tetracycline, nalidixic acid, penicillin G, and chloramphenicol were tested for *marRAB* phenotypic activity by growing isolates in the presence of 5 mM salicylate, a known modulator of the *marRAB* operon. These four antibiotics are associated with the *marRAB* operon, and were therefore used for *marRAB* induction assays. The results were compared to MICs of *E. coli* strains GC4468, AG112, and JHC1096 in which the *marRAB* operon was intact (wild-type), *marR* was deleted, or the entire *marRAB* operon was deleted, respectively [12,18,21]. With the exception of resistance to penicillin, in most cases the isolates were more resistant than the *marRAB*-deleted negative control, AG100, and less resistant than the constitutive *marRAB* mutant positive control, AG112 (data not shown).

No Pathogenic *E. coli* Strains Detected by PCR

Polymerase chain reaction was performed using primer pairs designed to detect *ial*, *estA*, *eltB*, *eaeA*, *invE*, *bfpA*, *stx₁* and *stx₂* virulence genes commonly associated with EPEC, EAEC, EIEC, ETEC, and STEC, pathogenic serotypes. After agarose gel electrophoresis, no bands were detected in any of the soil isolates, indicating that none of the dairy isolates were pathogenic. These results were compared to positive control DNA from *E. coli* pathotypes EPEC E2348/69, ETEC H10407, EIEC EDL1284 and STEC EDL933

Discussion

The relationship of dairy farm age and the prevalence of antimicrobial-resistance in *E. coli* was investigated in order to assess the ultimate effects of antibiotic residues in topsoil. Specifically, we investigated the role of the *marRAB* genetic locus in conferring resistance to *mar*-associated and non-*mar* associated antimicrobials in *E. coli* isolated from dairy farms of varying ages. *E. coli* were studied in particular because it is the predominant species isolated from the feces of dairy cattle [20]. Further, *E. coli* are poorly adapted to the soil and are unlikely to survive in the soil for longer periods.

There were no significant positive correlations between dairy farm age and *E. coli* resistance to chloramphenicol, nalidixic acid, tetracycline, and penicillin. This contradicts the hypothesis that higher antibiotic resistance would be observed in older dairy farms, as previous studies in food animal environments have shown positive correlations between antimicrobial usage and bacterial resistances [24].

General dairy farm practices might contribute to the observed lack of significant correlation between dairy age and antimicrobial susceptibility of *E. coli*. For instance, to reduce insect breeding grounds, it is not uncommon for farmers to use dirt and manure as bedding materials and to practice weekly corral scrapings to facilitate drying of bedding materials. Such maintenance of dairy grounds could potentially reduce the risk of bacterial infections by promoting proper drainage of contaminated water and reducing contact of cattle and humans with contaminated soil.

Antimicrobial agent use for growth and prophylactic purposes in agriculture and antimicrobial chemical properties may also contribute to the observed lack of correlation between dairy farm age and antimicrobial susceptibility of *E. coli*. Neither nalidixic acid nor chloramphenicol are used in dairy farms for fears of contribution to high-level resistance in humans or of disrupting protein synthesis in human bone marrow cells which can ultimately lead to aplastic and hypoplastic anemias, respectively [19]. Previous studies have shown a direct relationship between the decrease of antibiotic use and antibiotic resistance [1,9]. On the other hand, a study by Bunner et al. [3] showed that cessation of antimicrobial use did not immediately lower the levels of antimicrobial-resistant *E. coli* on swine farms.

The resistance to penicillin G is intrinsic in *E. coli* and in our study, penicillin G resistance was only studied in the context of *marCRAB* phenotype. The absence of any significant positive correlation observed between dairy farm age and the penicillin resistance can be due to inherent instability of the antibiotic as well as to the constant antibiotic deactivation by soil amidases [11]. Such factors would impede high selective pressures by penicillin and thus would not lead to higher levels of penicillin resistance.

Fluoroquinolones such as ciprofloxacin and nalidixic acid are less frequently applied in dairy cattle than in other species such as poultry [17]. This difference may explain the observation that in our study, no *E. coli* isolate was resistant to ciprofloxacin or nalidixic acid. We would expect that the *E. coli* isolates used in this study would not show

fluoroquinolone resistance, as resistance to naladixic acid is frequently used as an indicator of fluoroquinolone resistance. Some environment-antibiotic interactions make the correlation between farm age and resistance unlikely. In the case of tetracycline, it is easily inactivated upon direct exposure to sunlight. Thus, the limited availability of tetracycline in topsoil can perhaps lead to a decrease in selective pressure on the bacteria, thereby not allowing higher levels of resistance to be achieved over time. The chronic exposure of topsoil to tetracycline may cause the low-level resistance seen in Figure 1A. As with the high-level resistant isolates seen in a previous study [4], we suggest that the high-level resistant *E. coli* isolates seen in Figures 1–3 result from a step-wise evolution of resistance by accumulation of chromosomal or extra-chromosomal resistance determinants, yet their resistance may not be attributed to the age of the dairy farm.

The lack of any significant correlation between dairy farm age and *E. coli* resistance to chloramphenicol, nalidixic acid, tetracycline, ampicillin, amoxicillin/clavulanic acid, gentamicin, trimethoprim/culfamethoxazole, cefotaxime, and ciprofloxacin indicate that dairy farm topsoil does not support our hypothesis that dairy farm top soil is a reservoir of high-level antibiotic resistant strains evolved over time. Should antimicrobial-contaminated residues or antimicrobial-resistant organisms enter the food chain, undesirable consequences may ensue, such as increasing the prevalence of antibiotic-resistant bacteria in the human gut, or altering the protective barrier against infection provided by commensal gut flora [13]. Understanding the antibiotic resistant mechanisms in soil of food animal environments is critical as resistance that is overlooked can be easily transferred to other geographical regions. Thus, this study provides evidence that long-term use of antimicrobials in older dairy farms does not significantly affect antimicrobial resistance compared to the levels of bacterial resistance in younger farms.

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Figure 1.

Susceptibilities of dairy soil isolates to clinically- and veterinary- relevant antibiotics (n/R = number tested/number resistant). **A.** Dairy farm age and *E. coli* resistance to tetracycline. With the exception of five of 33 *E. coli* isolates, all isolates conferred low-level resistance to tetracycline. A positive but not significant correlation between dairy farm age and *E. coli* resistance to tetracycline was observed ($r_s=0.125$, $n=33$, $p>0.05$). **B.** Dairy farm age and *E. coli* resistance to chloramphenicol. With the exception of one of 38 *E. coli* isolates, all isolates conferred low-level resistance to chloramphenicol. A positive but not significant correlation between dairy farm age and *E. coli* resistance to chloramphenicol was observed ($r_s=0.238$, $n=38$, $p>0.05$). **C.** Dairy farm age and *E. coli* resistance to penicillin. With the exception of five of 34 strains that conferred high-level resistance, all isolates conferred low-level resistance to penicillin. A positive and but not significant correlation between dairy farm age and *E. coli* resistance to penicillin was observed ($r_s=0.495$, $n=34$, $p<0.0025$). **D.** Dairy farm age and *E. coli* resistance to nalidixic acid. With the exception of one of 37 *E. coli* isolates, all isolates conferred low-level resistance to nalidixic acid. A positive but not significant correlation between dairy farm age and *E. coli* resistance to nalidixic acid was observed ($r_s=0.107$, $n=37$, $p>0.05$).

Figure 2.

Susceptibilities of dairy soil isolates to clinically- and veterinary- relevant antibiotics. **A.** Dairy farm age and *E. coli* resistance to ciprofloxacin. A positive but not significant correlation between dairy farm age and *E. coli* resistance to ciprofloxacin was observed ($r_s=0.0740$, $n=41$, $P>0.05$). **B.** Dairy farm age and *E. coli* resistance to ampicillin. A positive but not significant correlation between dairy farm age and *E. coli* resistance to ampicillin was observed ($r_s=0.130$, $n=43$, $P>0.05$). **C.** Dairy farm age and *E. coli* resistance to amoxicillin/clavulanic acid. A negative and not significant correlation between dairy farm age and *E. coli* resistance to amoxicillin/clavulanic acid was observed ($r_s=-0.115$, $n=43$, $P>0.05$). **D.** Dairy farm age and *E. coli* resistance to gentamicin. A positive but not significant correlation between dairy farm age and *E. coli* resistance to gentamicin was observed ($r_s=0.204$, $n=42$, $p>0.05$).

Figure 3. Susceptibilities of dairy soil isolates to clinically- and veterinary- relevant antibiotics. **A.** Dairy farm Age and *E. coli* resistance to trimethoprim/sulfamethoxazole. A positive but not significant correlation between dairy farm age and *E. coli* resistance to trimethoprim/sulfamethoxazole was observed ($r_s=0.352$, $n=23$, $p>0.05$). **B.** Dairy farm age and *E. coli* resistance to cefotaxime. A negative but not significant correlation between dairy farm age and *E. coli* resistance to cefotaxime was observed ($r_s=-0.0211$, $n=42$, $P>0.05$).

Table 1

PCR primers used in this study

Target gene	Primer sequence	Product size	Reference
<i>aggR</i>	catctcttgataagtccttctcg gtatacacaaaagaaggaagc	434	26
<i>bfpA</i>	ggctctgtatattggcgagacc gccgctttatccaacctggta	347	31
<i>eaeA</i>	cccggcacaagacataagctaa tcgccgttcagactcgc	376	10
<i>ial</i>	ctggtaggtatgggtgagg ggaggccaacaattatttc	320	33
<i>stx1/2</i>	gagcgaataatttatatgtg tgatgatggcaattcagtat	404	16
<i>estA</i>	gctaaaccagtagggtcttcaaaa cccgtacaggcaggattacaaca	147	33
<i>eltB</i>	tctctatgtgcatacggagc ccatactgattgccgcaat	322	10

Table 2Susceptibility of *E. coli* isolates to non-*marRAB* associated antibiotics.

Antibiotic	Dairy Age in Years			
	0.5	7	10	24
Ampicillin (n=43)	1.412 ± 0.2121	0.153 ± 0.206	3.013 ± 0.957	1.434± 0.171
Amoxicillin/Clavulanic acid (n=43)	2.182 ± 0.205	1.487 ± 0.283	1.204 ± 0.226	1.817 ± 0.222
Cefotaxime (n=42)	0.0978 ± 0.020	0.089 ± 0.012	0.104 ± 0.048	0.0892± 0.014
Ciprofloxacin (n=41)	0.0135 ± 0.002	0.0086 ± 0.001	0.0118±0.003	0.0218 ±0.008
Gentamicin (n=42)	0.174 ± 0.014	0.118 ± 0.010	0.107 ± 0.006	0.230 ± 0.039
Trimethoprim/sulfamethoxazole (n=23)	0.0367 ± 0.010	0.0525 ± 0.011	0.0585 ± 0.035	0.0867± 0.027

The MIC results are expressed as mean ± SE of the mean. All isolates tested in this study were sensitive to non-*marRAB* antibiotics.