Published March 1, 2016

Journal of Environmental Quality SPECIAL SECTION

ANTIBIOTICS IN AGROECOSYSTEMS: STATE OF THE SCIENCE

Quantifying Attachment and Antibiotic Resistance of *Escherichia coli* from Conventional and Organic Swine Manure

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Abstract

Broad-spectrum antibiotics are often administered to swine, contributing to the occurrence of antibiotic-resistant bacteria in their manure. During land application, the bacteria in swine manure preferentially attach to particles in the soil, affecting their transport in overland flow. However, a quantitative understanding of these attachment mechanisms is lacking, and their relationship to antibiotic resistance is unknown. The objective of this study is to examine the relationships between antibiotic resistance and attachment to very fine silica sand in *Escherichia coli* collected from swine manure. A total of 556 isolates were collected from six farms, two organic and four conventional (antibiotics fed prophylactically). Antibiotic resistance was quantified using 13 antibiotics at three minimum inhibitory concentrations: resistant, intermediate, and susceptible. Of the 556 isolates used in the antibiotic resistance assays, 491 were subjected to an attachment assay. Results show that *E. coli* isolates from conventional systems were significantly more resistant to amoxicillin, ampicillin, chlortetracycline, erythromycin, kanamycin, neomycin, streptomycin, tetracycline, and tylosin ($P < 0.001$). Results also indicate that *E. coli* isolated from conventional systems attached to very fine silica sand at significantly higher levels than those from organic systems ($P < 0.001$). Statistical analysis showed that a significant relationship did not exist between antibiotic resistance levels and attachment in *E. coli* from conventional systems but did for organic systems ($P < 0.001$). Better quantification of these relationships is critical to understanding the behavior of *E. coli* in the environment and preventing exposure of human populations to antibiotic-resistant bacteria.

Core Ideas

- Greater levels of attachment were found in *E. coli* from conventional swine systems.
- A significant relationship exists between resistance and attachment for organic swine systems.
- Greater resistance to 9 of 13 antibiotics was found in conventional swine system *E. coli*.

J. Environ. Qual. 45:609–617 (2016)
doi:10.2134/jeq2015.05.0245
Received 28 May 2015.
Accepted 26 Jan. 2016.
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The US swine industry is a lucrative business. Iowa ranked first in conventional pork production in the United States in 2014, producing more than 20.9 million hogs and pigs with a collective value of approximately $6.8 billion (USDA–NASS, 2014). An additional $2.67 million was added to Iowa's economy by the production and sale of organically produced swine (USDA–NASS, 2011). According to some estimates, antibiotics were added to the diets of approximately 40% of swine produced on small-enterprise operations (<100 pigs) regardless of inventory class (i.e., sows, gilts, boars and young males for breeding, pigs not weaned and market pigs) (USDA Animal and Plant Health Inspection Service, 2012). In 2006, 88% of swine were fed antibiotics, an increase of 13% from 2002 (USDA Animal and Plant Health Inspection Service, 2002, 2006).

Only a fraction of the antibiotics administered orally is metabolized in vivo; upward of 75% may pass through the animal unchanged or with little alteration in chemical structure (Chee-Sanford et al., 2009). Swine manure and slurry is often stored in collection pits and lagoons and is periodically land applied, serving as a nutrient source for plants. This practice can lead to contamination of ground and surface waters, soils, and crops by antibiotics and antibiotic-resistant bacteria (ARB) (Jindal et al., 2006; Chee-Sanford et al., 2009) and may pose a risk to human health. Once in the environment, antibiotics can apply selective pressure to bacteria, contributing to the development and dissemination of antibiotic resistance among and within populations of bacteria (Sayah et al., 2005; Parveen et al., 2006; Sapkota et al., 2007). Antibiotic resistance in the environment can arise via spontaneous mutations in the bacterial genome and from the application of antibiotics for human health (Perez-Trallero et al., 2001; King et al., 2002). However, ARB are also found naturally

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Abbreviations: AMP, ampicillin; AMX, amoxicillin; ARB, antibiotic-resistant bacteria; CMP, chloramphenicol; CTC, chlortetracycline; ERY, erythromycin; GEN, gentamycin; KAN, kanamycin; MIC, minimum inhibitory concentration; NAL, nalidixic acid; NARMS, National Antimicrobial Resistance Monitoring System; NEO, neomycin; OR, odds ratio; SMZ, sulfamethazine; STP, streptomycin; TET, tetracycline; TYL, tylosin.

in soil, and many bacteria produce antibiotics as a means of self-protection (D'Costa et al., 2006; D'Costa et al., 2007).

One important but poorly understood pathway for exposure to ARB is through water systems. *Escherichia coli* is an important fecal indicator bacteria currently used by the USEPA to determine if a risk to human health is present due to possible exposure to pathogens. Because of this, many studies have investigated the fate and transport of *E. coli* in the environment (USEPA, 2012). *Escherichia coli* has been shown to attach preferentially to particulate matter (Auer and Niehaus, 1993; Ling et al., 2002; Fries et al., 2006; Liao et al., 2015), aiding in the survival and transport of bacteria within the environment (Gerba and McLeod, 1976; Burton et al., 1987; Pommepuy et al., 1992). The attached fraction of *E. coli* from runoff from manure-amended fields has been reported to range from >1 to >49% (Muirhead et al., 2005; Soupir and Mostaghimi, 2011). Although Smyth et al. (1978) and Boerlin et al. (2005) stated that differences in the propensity for attachment might vary between pathogenic and nonpathogenic strains of *E. coli*, others found no related patterns in the sorption of *E. coli* exposed to antibiotics but rather that attachment is highly variable and related to environmental conditions (Luppens et al., 2008; Petrova and Sauer, 2012; Gallagher et al., 2013).

Although particle-mediated transport of fecal indicator bacteria in the environment is clearly important (Liao et al., 2015), studies examining relationships between attachment and antibiotic resistance are limited. Liu et al. (2011) evaluated 203 porcine isolates of *E. coli* in an effort to determine if a relationship between attachment to very fine silica sand (sand) and antibiotic resistance exists. The researchers found that attachment to sand is related to the presence of the Type I attachment factor and to resistance to streptomycin (STP), chlortetracycline (CTC), tetracycline (TET), and tylosin (TYL). In the same study, Type I, P-pili, and Ag43 were also reported to be associated with resistance to neomycin (NEO) and amoxicillin (AMX), leading to the hypothesis that encoding of resistance and virulence factors is likely due to a single mobile genetic element, such as a plasmid. Studies using clinical isolates in the presence of biomaterials have shown that antibiotic resistance and the presence of genes that encode for adhesion have been implicated in the persistence and infection rates by bacteria in humans (Hagberg et al., 1983; Wullt et al., 2000; Arisoy et al., 2008; Karami et al., 2008). Given the reported genetic relationship between genes encoding attachment and antibiotic resistance, it is not unexpected that such a relationship exists (Nowrouzian et al., 2001).

Data have been reported describing the attachment of bacteria to certain surfaces, and a few efforts have indicated a relationship between attachment and antibiotic resistance (Pachepsky et al., 2008; Liu et al., 2011). However, very little research has been conducted to examine these relationships when environmental isolates are collected from swine facilities with varying antibiotic use practices. The objectives of this study were (i) to detect and quantify the fraction of *E. coli* isolated from manure collected from conventional and organic swine production facilities in Iowa showing attachment to sand; (ii) to quantify the resistance (susceptible, intermediate, or resistant) levels of isolates collected from conventional and organic swine production facilities to AMX, ampicillin (AMP), chloramphenicol (CMP), CTC, erythromycin (ERY), gentamycin (GEN), kanamycin (KAN), nalidixic acid (NAL), NEO, TET, TYL, STP, and sulfamethazine (SMZ); and (iii) to analyze statistical relationships between antibiotic resistance and attachment under different management practices (conventional and organic).

Materials and Methods

Collection and Enumeration

Manure samples were collected from six farms. Two farms were managed organically and four were managed conventionally, where antibiotics were administered at subtherapeutic levels (Table 1). Samples were collected between the fall of 2008 and the spring of 2009. At four locations fresh manure samples were collected and at two locations samples were collected from a deep pit and lagoon. A subset of random resistant isolates from this study was further used in an attachment marker study by Liu et al. (2011).

Samples were collected in sterile 1-L plastic containers, transported, stored at <4°C, and processed within 6 h of collection. A mortar and pestle were used to homogenize samples from each location under sterile conditions, and 1 g of manure was mixed in 9 mL of phosphate-buffered water for 10 min, in triplicate. Serial dilutions were made, and *E. coli* were enumerated using USEPA Method 1603 (USEPA, 2009).

From each location, 100 typical (magenta in color on modified mTEC agar) and atypical (pale yellow) *E. coli* colonies were selected and grown individually in 2-mL test tubes containing a 1.5-mL glucose and 2% Luria-Bertani solution; glucose was added to ensure that growth was not limited. Stab inoculations of each strain were prepared for storage at 4°C in Luria-Bertani solution plus 1.5% agar and also separately maintained in 25% glycerol frozen stocks at −70°C (Liu et al., 2011). During the course of the experiments, control experiments were performed to ensure the viability and survivability of the *E. coli* and to ensure *E. coli* counts were maintained. In any case where the control did not hold (i.e., when contamination of the control sample was evident, the control did not grow, or some other issue caused an expected response from the control), the results from that strain were dropped or rerun.

Table 1. Description of research farms for manure collection used in selecting and enumeration of bacterial isolates.

Location	Farm type	Collection date	Waste management	Antibiotic practice	Reported antibiotic use
Ames	breeding	Nov. 2008	lagoon	conventional	NT-80†
Manning	finishing	Nov. 2008	deep pit	conventional	NT-80
Nashua	finishing	Apr. 2009	deep pit	conventional	Tylan
New Hampton	finishing	Apr. 2009	bedding	organic	none
Ames	farrowing	May 2009	deep pit	organic	none
Ames	finishing	May 2009	deep pit	conventional	Tylan

† A proprietary feed additive including chlortetracycline, sulfamethazine, and penicillin in combination and at times tylosin if needed.

Attachment

The approximate size of a single *E. coli* (0.5 μm × 2.5 μm) and the largest surface area per bacterium (1.5×10^6 mm^2) that could attach was used to calculate the mass of sand needed for each sample such that sand surface area was nonlimiting during the attachment assay. Very fine silica sand (74–177 μm) was rinsed in distilled water and dried at 105°C for 1 h. The mass of sand needed per sample was determined as described previously by Liu et al. (2011). Sand was added to 50-mL conical tubes using aseptic techniques.

Individual strains were aseptically transferred to 15-mL conical tubes containing Mueller Hinton broth (Difco) and placed for 12 h in a reciprocal shaking water bath (Thermo Scientific) at 37°C. Samples were removed from the water bath and centrifuged at 280 × *g* for 5 min in a refrigerated centrifuge (model 5702 R, Eppendorf) at 4°C. The supernatant was discarded. Ten milliliters of phosphate-buffered water was added to the remaining pellet, and bacteria were resuspended. Phosphate-buffered water was used to dilute bacterial cultures to a 0.5 McFarland standard (1.0×10^8 cfu mL^{-1}) (Clinical and Laboratory Standards Institute, 2006a).

Forty milliliters of the diluted bacteria cultures were aseptically added to the 50-mL conical tubes containing sand. The sand–bacteria suspensions were vortexed briefly to mix and immediately placed horizontally on an orbital shaker for 20 min at 80 rpm (Gallagher et al., 2013). After shaking, the conical tubes were placed vertically in racks, and the sand particles were allowed to settle via gravity for 5 min. Stokes' law was used to calculate the settling time of the sand in the attachment assay. For this calculation, a dynamic viscosity and density of water of 1.002×10^{-3} Pa s and 1000 kg m^{-3}, respectively, were used, as was an average particle radius of 3.7×10^{-5} m and a density of 2.43×10^{11} kg m^{-3} for sand. The resulting average settling velocity computed was 0.724 m s^{-1}.

After settling, 1 mL of supernatant was serially diluted to 1×10^6 using phosphate-buffered water. The total number of *E. coli* in the supernatant was determined using USEPA Method 1603 and was recorded as the unattached population. Control tubes indicated that reproduction was not significant in the 20-min incubation period, and controls were run to ensure that sorption of *E. coli* to the interior surface of the tubes used was not significant. A mass balance equation was used to evaluate the attached fraction of *E. coli* in each sample where the attached portion was assumed to be the difference between the unattached concentration in the supernatant and the total *E. coli* concentration (Soupir et al., 2010; Soupir and Mostaghimi, 2011). Percent attachment was calculated by dividing the attached number of cells by the total number of cells and converting to a percent. All positive values were assumed as attached.

Antibiotic Resistance

Antibiotics for this study were selected by comparing the USDA's list of antibiotics approved for swine with a National Antimicrobial Resistance Monitoring System (NARMS) World Health Organization list of antibiotics used in human medicine. Where the two lists corresponded, a representative antibiotic from six of the eight antibiotic modes of action was selected for screening. Antibiotic susceptibility was determined by agar dilution procedures (Clinical and Laboratory Standards Institute, 2006a) using standard powders of the following antimicrobial agents from Sigma Aldrich: AMX and AMP (penicillins); CTC and TET (tetracyclines); ERY and TYL (macrolides); GEN, KAN, NEO, and STP (aminoglycosides); NAL (quinolones); SMZ (sulfonamides); and CMP (other). Chloramphenicol is functionally similar to a macrolide in that it affects the 50S ribosomal subunit (Thompson et al., 2002). With the exception of TYL, CMP, and ERY, the antibiotics used in this study are important in the treatment of infections caused by Gram-negative bacteria such as *E. coli*; however, CMP is often carried on a plasmid that also codes for resistance to multiple antibiotics (Jorgensen, 1978) that are effective on Gram-negative bacteria, and ERY is effective against some Gram-negative bacteria (Mao and Putterman, 1968). Guidelines from the CLSI (formerly the National Committee for Clinical Laboratory Standards) were used to prepare and dilute all antimicrobial agents except TYL, which was dissolved in methanol and adjusted to pH 7.9 using 0.1 mol L^{-1} phosphate buffer (Kaukas et al., 1988). The antimicrobials were tested using susceptible, intermediate, and resistant minimum inhibitory concentrations (MICs) (Table 2). A total of 556 *E. coli* isolates were tested in triplicate, as were ATCC 29522 (a Gram-negative control strain) (Clinical and Laboratory Standards Institute, 2006b; Wiegand et al., 2008), *E. coli* K12 MG1655 (a wild-type strain), and MG1655 with pPAP plasmid (Arisoy et al., 2008). All antibiotics tested fell within the MIC quality control ranges established by the CLSI for *E. coli* ATCC 29522. Antibiotic dilution plates for the three reported MIC levels—susceptible, intermediate, and resistant—for each antibiotic were made and stored for 48 h at 4°C until inoculation. Before inoculation, plates were removed from the cooler and allowed to come to room temperature.

Individual strains were grown overnight in 10 mL of Mueller Hinton broth placed in 15-mL conical tubes at 37°C in a reciprocating shaker water bath (Thermo Scientific). Before inoculation of antibiotic dilution plates, strains were diluted to a 0.5

Table 2. Minimum inhibitory concentrations for antimicrobial agents tested as against 556 *Escherichia coli* isolates and three control isolates.†

Antimicrobial agent	Minimum inhibitory concentrations‡		
	Susceptible	Intermediate	Resistant
	μg mL^{-1}		
Amoxicillin	16	32	48
Ampicillin	16	32	48
Chloramphenicol	16	32	48
Chlortetracycline	16	32	48
Erythromycin	15	20	30
Gentamycin	8	16	24
Kanamycin	32	64	96
Nalidixic acid	16	32	48
Neomycin	8	16	24
Tetracycline	24	16	24
Tylosin	16	32	48
Streptomycin	12	15	22.5
Sulfamethazine	256	512	768

† Control isolates were ATCC 29522TM (a Gram-negative control strain), *E. coli* K12 MG1655 (wild-type), and MG1655 (positive for pPAP).

‡ Minimum inhibitory concentrations for all antibiotics except tylosin were set according to the Clinical and Laboratory Standards Institute (2006b). Tylosin was prepared according to Kaukas et al. (1988).

McFarland standard with phosphate-buffered water. One microliter of each strain was aseptically transferred to plates containing one of each of the 13 antimicrobial agents at the three MIC levels. All strains were tested in triplicate and plated on Mueller Hinton Agar and Mueller Hinton Agar II as controls per CLSI guidelines. Plates were allowed to dry and inverted for incubation at 37°C for 18 ± 2 h. Isolates were recorded as present or absent after incubation.

Statistical Analyses

Percent resistant and susceptible *E. coli* for each antibiotic was calculated as the ratio of resistant to the total number analyzed multiplied by 100. Strains found to be intermediately resistant were included as resistant bacteria in subsequent calculations. Statistical analyses were performed using SPSS (version 20) and SAS (version 9.2) software. Pairwise deletions were used in dealing with missing data, rather than imputation, because the total percentage of missing data was <5%, and imputation might have introduced bias into the results (Fink, 2006; McKnight et al., 2007; Tabachnick and Fidell, 2007). An odds ratio (OR) is a calculation of association between an exposure and an outcome. It represents the odds that a particular outcome will occur given a particular exposure compared with the odds of the outcome occurring without that exposure. Odds ratios are often used to compare the relative odds of the occurrence of that outcome of interest—in this case, attachment—given exposure to the variable of interest (antibiotic). An OR of 1 signifies that exposure does not affect the odds of outcome, an OR >1 indicates that exposure is associated with a higher odds of outcome, and an OR <1 signifies that exposure is associated with a lower odds of outcome (Szumilas, 2010). In this study, ORs were used in running the Breslow–Day and Cochran–Mantel–Haensel tests, which were run to test whether the statistical distribution of *E. coli* from management system and antibiotic resistance were the same for all antibiotics and whether a partial association was present between management system and level of resistance when controlling for antibiotic type, respectively.

Independent samples *t* test and χ^2 tests of independence were performed on the resulting data. Normality and equal variance were assumed for the independent samples *t* test. Levene's test was used to test for equal variance between the groups of conventionally and organically managed isolate groups for attachment. Because the variances were determined not to be equal and the equal variance assumption was not met ($P < 0.001$), an adjusted independent *t* test value was calculated and used. Chi-square tests of independence included adjusted standardized residuals for each value in the cross tabulation table. The adjusted standardized residual used was a z-score, a measurement of SD from the expected value of an actual value in the χ^2 contingency table (Sharpe, 2015). Therefore, adjusted standardized residuals of the absolute value of 3 or greater were considered to be contributing a significant amount to the χ^2 value, and those with an absolute value <3 were not (Agresti, 2002). SPSS v.20 was used to perform *t* tests and χ^2 tests of independence, and SAS v9.2 was used to perform the Breslow–Day and the Cochran–Mantel–Haensel omnibus tests. A 95% level of significance (significance at $P < 0.05$) was set for all analyses.

Results and Discussion

Attachment

A total of 491 isolates were used in the attachment assays. Any isolate that was collected and used for the resistance assays that was not viable was not used in this portion of the study. Conventionally managed systems accounted for 62.5% of all records, and organically managed systems accounted for 37.5%. Results from the Breslow–Day test indicate that there were significant differences in bacterial attachment among the 13 antibiotics ($P < 0.0001$). Results from the Cochran–Mantel–Haensel procedure ($P < 0.0001$) indicated that a partial association was present between management system and level of resistance when controlling for antibiotic type.

Escherichia coli isolates from conventionally managed swine systems showed significantly higher ($P < 0.001$) levels of attachment overall when compared with those from organically managed systems. The conventionally managed group had a greater proportion of attached isolates than expected, whereas the organically managed isolates had a lesser number than expected of attached isolates (Tables 3 and 4). As described above, significance was assessed based on the value of the standard residual, with absolute values >3 being classified as significant. For the organically managed system, the standard residual was not

Table 3. Cross tabulation of isolate attachment versus resistance level for isolates assayed under the conventional management system including controls, in triplicate.

Attachment	Resistance level			Total
	Susceptible	Intermediate	Resistant	
Attached	1039	126	1469	2634
Expected count	1060.7	128.0	1445.3	–
% Total	26.6	3.2	37.6	67.3
Adj. std. residual†	−1.5	−0.3	1.6	–
Unattached	536	64	677	1277
Expected count	514.3	62.0	700.7	–
% Total	13.7	1.6	17.3	32.7
Adj. std. residual	1.5	0.3	−1.6	–
Total	1575	190	2146	3911
Expected count	–	–	–	–
% Total	40.3	4.9	54.9	100.0

$\chi^2(2) = 2.647$; $P = 0.266$

† Adjusted standardized residual.

Table 4. Cross tabulation of isolate attachment versus resistance level for isolates assayed under the organic management system including controls, in triplicate.

Attachment	Resistance level			Total
	Susceptible	Intermediate	Resistant	
Attached	686	70	547	1303
Expected count	644.6	71.7	586.7	–
% Total	29.1	3.0	23.2	55.2
Adj. std. residual†	3.4	−0.3	−3.3	–
Unattached	482	60	516	1058
Expected count	523.4	58.3	476.3	–
% Total	20.4	2.5	21.9	44.8
Adj. std. residual	−3.4	0.3	3.3	–
Total	1168	130	1063	2361
Expected Count	–	–	–	–
% Total	49.5	5.5	45.0	100.0

$\chi^2(2) = 12.009$; $P = 0.002$

† Adjusted standardized residual.

greater than the absolute value of 3, and therefore the value did not contribute significantly to the χ^2 value, whereas the standard residual for the conventionally managed system was found to be contributing a significant amount (Tables 3 and 4). Table 5 shows yet another comparison of resistance and attachment among the 491 isolates screened in the attachment assay. For example, 123 (80%) of the AMX-resistant isolates were from conventionally managed systems, and 30 (20%) were from organically managed systems. Of the 153 AMX-resistant isolates from conventional and organic systems, 83 and 17%, respectively, attached to the very fine silica sand. Resistant bacteria from 12 (AMX, AMP, CMP, ERY, GEN, KAN, NEO, TET, TYL, STP, and SMZ) of the 13 antibiotics tested exhibited greater levels of attachment under conventional management systems (Table 5). In the case of NAL, 55% of resistant isolates from organic management systems attached to sand, whereas only 45% of the resistant isolates from conventionally managed systems attached (Table 5).

Differences in attachment among isolates, although significant, were not surprising. Research has been performed in recent years to elucidate the factors contributing to or limiting bacterial attachment in a porous media such as sand. These factors include, but are not limited to, cellular dynamics such as conditions for growth (Walker et al., 2005; Yang et al., 2006), composition of the polymeric compounds in the cell matrix (Haznedaroglu et al., 2008; Bolster et al., 2009), cellular surface charge and hydrophobicity (Lutterodt et al., 2009; Foppen et al., 2010; Bolster et al., 2010; Walczak et al., 2012), and the presence of genetic attachment factors and motility (Yang et al., 2008; Lutterodt et al., 2009; Liu et al., 2011). Other important factors include acclimation time of cells (Castro and Tufenkji, 2007; Haznedaroglu et al., 2008), temperature (Castro and Tufenkji, 2007), ionic strength (Bolster et al., 2006), and media characteristics such as particle size and composition (Bradford et al., 2006; Bolster et al., 2009), hydraulic conductivity (Levy et al., 2007), and moisture content (Foppen and Schijven, 2006; Jiang et al., 2007).

Antibiotic Resistance

The frequency of isolates and their corresponding level of resistance to each of the 13 antibiotics are presented (Table 6) (n = 6383). Overall, more than half (54%) of the records (n =

Table 5. Relative impact of conventional or organic management on antibiotic resistance to specified antibiotics and attachment to very fine silica sand. A total of 491 isolates were assayed for attachment to very fine silica sand and resistance to antibiotics from organic and conventionally managed swine systems.

	Resistant				Attached†			
	Conventional		Organic		Conventional		Organic	
	n	%	*n*	%	*n*	%	*n*	%
Amoxicillin	123	80	30	20	81	83	17	17
Ampicillin	137	74	47	26	88	78	25	22
Chloramphenicol	53	57	40	43	40	68	19	32
Chlortetracycline	245	70	105	30	166	76	51	24
Erythromycin	282	67	140	33	194	72	76	28
Gentamycin	48	57	36	43	35	70	15	30
Kanamycin	157	71	63	29	106	73	40	27
Nalidixic acid	23	31	51	69	20	45	24	55
Neomycin	98	71	41	29	69	76	22	24
Tetracycline	272	65	144	35	186	72	73	28
Tylosin	288	64	162	36	195	69	86	31
Streptomycin	217	74	78	26	144	79	39	21
Sulfamethazine	203	62	125	38	145	71	60	29

† The attached portion was assumed to be the difference between the unattached and total *Escherichia coli* concentrations.

2146) for conventional systems were classified as resistant, and, among isolates from organic systems, a greater percentage was classified as susceptible (49%; $n = 1168$) than resistant (44%; $n = 1063$). The total percentage of isolates resistant to more than one antibiotic was also tabulated for the 556 isolates tested (Table 7). All isolates—despite management system classification—found to be resistant displayed resistance to more than one antibiotic. For example, of the 557 isolates tested, 88 isolates (15%) were resistant to AMX, and of those AMX-resistant isolates 74 isolates (88%) were also resistant to AMP and 76 isolates (90%) were also resistant to CMP.

To investigate differences in the counts of the three resistance levels to antibiotics for isolates by management system, a series of χ^2 tests of independence were performed. One χ^2 test of independence was performed for each of the 13 antibiotic types (Table 6). Results indicate that *E. coli* isolates from manure produced under conventional management swine systems exhibited higher levels of resistance to AMX, AMP, CTC, ERY, KAN, NEO, STP, TET, and TYL. However, χ^2 tests of independence were not statistically significant for CMP, GEN, and SMZ. Additionally, the analysis of isolates for NAL resistance levels indicated that the conventional management system had a significantly smaller than expected proportion of resistant isolates.

Nalidixic acid belongs to a class of antibiotics that has historically been reserved for use on more resistant strains of bacteria (Emmerson and Jones, 2003). The frequency of resistance to NAL found in this study (31%) was greater than that reported in a 2005 study (9.3%) conducted by NARMS (NARMS, 2005). In a more recent NARMS report (NARMS, 2012), the frequency of resistance to NAL was much lower; however, the reported values are for *E. coli* O157 only and not for all *E. coli* isolates, as in 2005. The current study also found that the frequencies of resistance to AMX and KAN were significantly higher than those reported by NARMS (31.2 vs. 4.2% and 44.2 vs. 0%, respectively). Interestingly, the criteria used for determining antibiotic resistance here were more rigorous than those used in the NARMS report (NARMS, 2005). Antibiotic resistance results from this study (GEN, 17%; TET, 85%; ERY, 86%) are consistent with findings by Moore et al. (2010), who reported similar resistance levels of GEN (17%), TET (75%), and ERY (75%).

For most antibiotic groups, a majority of records were classified as susceptible for both conventional and organic management (Table 6). Chlortetracycline was the exception, with 80% of the isolates testing classified as resistant from the conventionally managed system, whereas only 57% of the isolates from the organically managed system were resistant. Given the prolific therapeutic and subtherapeutic use of tetracyclines and their ubiquitous presence in soil, this finding was not surprising. To ensure the effectiveness of this class of antibiotics going

Table 6. Chi-square tests of independence were performed to investigate differences in the number of isolates for each of the three levels of resistance to the 13 antibiotics for isolates, by management system (conventional vs. organic).

Antibiotic	Management system	*n*†	Resistance level frequency‡			χ^2	*P* value
			S	I	R		
Amoxicillin	conventional	307	178 (58)§	6 (2)	123 (40)	30.28	<0.0005
	organic	184	149 (81)	5 (3)	30 (16)		
Ampicillin	conventional	307	161 (52)	9 (3)	137 (45)	18.02	<0.005
	organic	184	131 (71)	6 (3)	47 (26)		
Chloramphenicol	conventional	307	236 (77)	18 (6)	53 (17)	2.26	0.323
	organic	184	137 (74)	7 (4)	40 (22)		
Chlortetracycline	conventional	307	55 (18)	7 (2)	245 (80)	28.3	<0.005
	organic	183	70 (38)	8 (4)	105 (57)		
Erythromycin	conventional	307	16 (5)	9 (3)	282 (92)	41.65	<0.005
	organic	184	44 (24)	0 (0)	140 (76)		
Gentamycin	conventional	307	250 (81)	9 (3)	48 (16)	2.12	0.347
	organic	184	140 (76)	8 (4)	36 (20)		
Kanamycin	conventional	301	127 (42)	17 (6)	157 (52)	20.16	<0.005
	organic	184	116 (63)	5 (3)	63 (34)		
Nalidixic acid	conventional	291	223 (77)	45 (15)	23 (8)	36.74	<0.005
	organic	184	117 (64)	16 (9)	51 (28)		
Neomycin	conventional	307	174 (57)	35 (11)	98 (32)	11.57	0.003
	organic	184	132 (72)	11 (6)	41 (22)		
Streptomycin	conventional	273	35 (13)	21 (8)	217 (79)	57.59	<0.0005
	organic	169	36 (21)	54 (32)	79 (47)		
Sulfamethazine	conventional	262	54 (21)	5 (2)	203 (77)	0.01	0.994
	organic	169	41 (24)	3 (2)	125 (74)		
Tetracycline	conventional	307	30 (10)	5 (2)	272 (89)	9.65	0.008
	organic	184	33 (18)	7 (4)	144 (78)		
Tylosin	conventional	307	15 (5)	4 (1)	288 (94)	10.45	0.005
	organic	184	22 (12)	0 (0)	162 (88)		

† Total number of isolates by management system tested for each antibiotic.

‡ I, intermediate; R, resistant; S, susceptible.

§ Values in parentheses are percentage of isolates by management system within resistance level.

Table 7. Number of isolates displaying resistance to each of the 13 antibiotics tested.†

Antibiotic	Amoxicillin	Ampicillin	Chloramphenicol	Chlortetracycline	Erythromycin	Gentamycin	Kanamycin	Nalidixic acid	Neomycin	Tetracycline	Tylosin	Streptomycin	Sulfamethazine
Amoxicillin	84 (15)‡												
Ampicillin	74 (88)	184 (33)											
Chloramphenicol	76 (90)	165 (90)	461 (83)										
Chlortetracycline	65 (77)	127 (69)	306 (66)	328 (59)									
Erythromycin	49 (58)	116 (63)	187 (41)	159 (48)	220 (40)								
Gentamycin	84 (100)	172 (93)	447 (97)	322 (98)	131 (60)	444 (80)							
Kanamycin	76 (90)	174 (95)	370 (80)	289 (88)	196 (89)	399 (90)	399 (72)						
Nalidixic acid	51 (61)	139 (76)	131 (28)	100 (30)	82 (37)	141 (32)	139 (35)	170 (31)					
Neomycin	69 (82)	159 (86)	340 (74)	263 (80)	166 (75)	341 (77)	313 (78)	124 (73)	325 (58)				
Tetracycline	44 (52)	85 (46)	127 (28)	103 (31)	125 (57)	138 (31)	131 (33)	57 (34)	116 (36)	139 (25)			
Tylosin	29 (35)	39 (21)	58 (13)	47 (14)	43 (20)	65 (15)	67 (17)	29 (17)	40 (12)	25 (18)	119 (21)		
Streptomycin	75 (89)	152 (83)	287 (62)	238 (73)	162 (74)	285 (64)	275 (69)	115 (68)	261 (80)	116 (83)	39 (33)	278 (50)	
Sulfamethazine	59 (70)	73 (40)	90 (20)	77 (23)	56 (25)	92 (21)	87 (22)	51 (30)	84 (26)	52 (37)	28 (24)	78 (28)	93 (17)

† The numbers of isolates that are resistant to a given antibiotic but are also resistant to each of the other 12 antibiotics are shown. For example, of the 556 isolates tested, 84 (15%) were resistant to amoxicillin; of those 84 isolates, 74 (88% of 84), 76 (90% of 84), and 65 (77% of 84) were also resistant to ampicillin, chloramphenicol, and chlortetracycline, respectively. Numbers should be read down the column only.

‡ Values in parentheses are percentage of the total (n = 556).

forward, research has been performed to identify inhibitors of tetracycline resistance and to identify new antibiotic classes for use in treatment (Nelson et al., 1993; Nelson and Levy, 1999). A majority of isolates from both the conventional and organic management systems were classified as resistant to ERY (92 and 76%, respectively) and TYL (94 and 88%, respectively) (Table 6). Erythromycin is a macrolide antibiotic that is normally used to treat infections caused by Gram-positive bacteria. This class of antibiotics is not normally active against Gram-negative bacteria due to the antibiotic structure, which includes large hydrophobic molecules that are not likely to penetrate both the inner and outer membranes of Gram-negative bacteria (Pai et al., 2000). Resistance has, however, been found in *Shigella* ssp., which is closely related to *E. coli* and which may readily exchange plasmids with specific phylogenetic groups of *E. coli* (Bezuidt et al., 2011).

Antibiotic Resistance and Attachment by Management

As previously discussed herein, two omnibus tests were performed to investigate the overall effects for each of the 13 antibiotics. Results from the Breslow–Day procedure—testing that the ORs for management system versus level of resistance were the same for both management systems—were statistically significant (P = 0.001). Therefore, there was sufficient evidence to indicate the ORs differed between the two management groups for the 13 antibiotics. The Cochran–Mantel–Haensel procedure was used to test whether there was evidence of partial association between attachment and level of resistance when controlling for management system. The test was not statistically significant (P = 0.290); therefore, no evidence for a partial association existed.

To investigate differences in the number of bacteria at each MIC level per antibiotic by attachment classification, a series of χ^2 tests of independence was performed—one for each of the two management system types. No significant relationship between level of resistance and isolate attachment for the conventional management system was found (P = 0.266), indicating that there were no significant differences in the proportions of isolates resistant to antibiotics for the isolate attachment groups (Table 3). However, significant differences were detected between the level of resistance and isolate attachment for the organic management system (P = 0.002) (Table 4). The unattached and attached groups both had a greater proportion of resistant-level isolates than expected. Although the mechanism(s) responsible for the correlation between antibiotic resistance and attachment were not investigated for this paper, Liu et al. (2011) reported a significant correlation between the Type I attachment factor and resistance to STP, CTC, TET, and TYL on a subset of the isolates used in this study. A correlation was also reported between AMP resistance and the presence of the P pili attachment factor (Liu et al., 2011). These results may support the supposition that resistance and attachment factors could be included on the same mobile genetic element. In studies using clinical isolates, ARB have been shown to preferentially attach due to the coupling of resistance genes and attachment factors on the same mobile genetic elements, such as a genetic cassette (Gallant et al., 2005; Teodosio et al., 2012). However, Teh et al. (2014) noted that the number of virulence genes carried by isolates may not directly affect their ability to attach or form biofilms.

Although many swine systems in the United States continue to add broad-spectrum antibiotics for growth promotion and prophylaxis, the frequency of this practice has declined in recent years due to a shift in public opinion. Results from this study indicate that further research is needed to understand the genetic relationship between bacterial attachment factors and antibiotic resistance as well elucidation of the mechanism(s) behind resistance to multiple classes of antibiotics. Such research will be crucial so that best management recommendations can be made to farmers regarding the timing and placement of manure to fields.

Acknowledgments

Funding for this work was provided by Iowa State University. The authors thank Bridget Huss, Molly Zwonitzer, Pagie Dugal Arve, and David Westhoff for their assistance with sample collection and analysis; ISU research farms and other private farms for providing access to manure samples; and Elaine Eisenbeisz, Heather Torres, and Heather Crutcher at Omega Statistics for help in analyzing these datasets.

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