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TECHNICAL NOTES & PRELIMINARY COMMUNICATIONS

Antibiotic resistance gene profile changes in cropland soil after manure application and rainfall

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Abstract

Land application of manure introduces gastrointestinal microbes into the environment, including bacteria carrying antibiotic resistance genes (ARGs). Measuring soil ARGs is important for active stewardship efforts to minimize gene flow from agricultural production systems; however, the variety of sampling protocols and target genes makes it difficult to compare ARG results between studies. We used polymerase chain reaction (PCR) methods to characterize and/or quantify 27 ARG targets in soils from 20 replicate, long-term no-till plots, before and after swine manure application and simulated rainfall and runoff. All samples were negative for the 10 b-lactamase genes assayed. For tetracycline resistance, only source manure and post-application soil samples were positive. The mean number of macrolide, sulfonamide, and integrase genes increased in post-application soils when compared with source manure, but at plot level only, 1/20, 5/20, and 11/20 plots post-application showed an increase in *erm*(B), *sulI*, and *intI*1, respectively. Results confirmed the potential for temporary blooms of ARGs after manure application, likely linked to soil moisture levels. Results highlight uneven distribution of ARG targets, even within the same soil type and at the farm plot level. This heterogeneity presents a challenge for separating effects of manure application from background ARG noise under field conditions and needs to be considered when designing studies to evaluate the impact of best management practices to reduce ARG or for surveillance. We propose expressing normalized quantitative PCR (qPCR) ARG values as the number of ARG targets per 100,000 16S ribosomal RNA genes for ease of interpretation and to align with incidence rate data.

1 | INTRODUCTION

The urgency and complexity of the global health crisis caused by antibiotic resistance is driving interest in understanding the prevalence, fate, and transport of agricultural and environmental antibiotic resistance. Soil is a natural

Abbreviations: ARG, antibiotic resistance gene; LOD, limit of detection; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal RNA.

reservoir of antibiotic-resistant bacteria, and their genes can be found in soils across the globe (Cytryn, 2013; D'Costa, McGrann, Hughes, & Wright, 2006; Durso, Miller, & Wienhold, 2012). Human and animal feces are also a natural reservoir of antibiotic resistance genes (ARGs) (Stanton, Humphrey, & Stoffregen, 2011), and ARGs are enriched from individuals that receive antibiotics (Looft et al., 2012).

The application of animal manures to soil is an ancient practice (Bogaard, Heaton, Poulton, & Merbach, 2007), which continues today in both conventional and organic farming systems. Manure provides valuable nutrients for crops, increases soil organic matter and improves soil health (Doran & Zeiss, 2000; Edmeades, Thorrold, & Roberts, 2005; Garcia-Pausas, Rovira, Rabissi, & Romanyà, 2017). It also introduces gastrointestinal microbes into the environment, raising concerns related to infectious disease and antibiotic resistance (Marti et al., 2013; McKinney, Dungan, Moore, & Leytem, 2018).

The details of manure-borne ARG persistence in soils after land application remain unclear, with some studies reporting temporal declines reaching background levels over the course of a growing season (Chen et al., 2017; Durso, Miller, & Henry, 2018; Marti et al., 2013, 2014), whereas others report increases in specific ARG targets (Scott, Tien, Drury, Reynolds, & Topp, 2018). In one study, variable results were seen for *sulI* (sulfonamide), *erm*(B) (erythromycin), and *intI1* (Class 1 integron-integrase) genes immediately after land application of manures, where both exponential decay and increases were observed in the short-term during different years (Marti et al., 2014).

Deciphering the impact of manured soils on measures of antibiotic resistance in the environment is complicated by large areas to be tested, generally limited replication in field studies, and the known heterogeneity of soil systems (Kelley, 1922; Pepper & Brusseau, 2019). To explore the repeatability of field ARG measurements, we used a before-and-after framework common in land application studies to examine 20 replicate field plots receiving manure and simulated rainfall. We expected the ARG dynamics in all 20 plots to be similar, and pre-application soil ARG measurements to be lower than ARG measurements of recently manured soils.

Core Ideas

- There is uneven distribution of ARGs even in replicate plots of the same soil type and on the same farm.
- Temporary blooms of ARGs after manure application were likely linked to soil moisture levels.
- Heterogeneity of ARG distribution should be considered when planning environmental surveillance.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Soil samples (0–10 cm) were collected from plots at the University of Nebraska Rogers Memorial Farm (40°50′42″ N, 96°28′19″ W) (Gilley et al., 2017). Twenty no-till plots were established, perpendicular to the slope, on fields documented to have received no manure application since the farm was bequeathed to the university in 1947. The fields had previously been used for winter wheat (*Triticum aestivum* L.), soybean [*Glycine max* (L.) Merr.], corn (*Zea mays* L.), and grain sorghum [*Sorghum bicolor* (L.) Moench], with winter wheat harvested from the study site the previous year. The wheat residue was not chopped and provided 100% soil coverage. The Aksarben clay loam (fine, smectic, mesic Typic Arguidolls) was representative of soils found in southeastern Nebraska (Supplemental Table S1). Replicate plots were sampled at two time points, 1 wk apart (Supplemental Figure S1). Due to the labor-intensive nature of the field collection protocol involving simulated rainfall over the 20 plots (Gilley et al., 2017), two plots were processed each week during a 10-wk test period, with new manure applied weekly ($n = 10$ source manures).

Swine manure was collected weekly from a commercial deep pit swine operation in southeast Nebraska and transported in 20-L plastic buckets. An initial subsample of manure slurry was collected prior to the start of experiments and characterized for chemical and physical properties (Gilley et al., 2017). These values were used to calculate the application rate, with an estimated target of 151 kg N ha^{-1} yr^{-1}, the rate needed to meet the annual N requirement for corn. Functionally, the addition of manure adds not only nutrients, but also antibiotic-resistant bacteria and ARGs to the soil. The slurry was surface applied by hand at a rate of 3.90×10^4 kg ha^{-1}, simulating a typical manure spreader. The manure was left on the soil surface without incorporation via tillage or other methods.

Twenty pre-application and 20 post-application samples were each collected from a 10-cm^3 area that was 20 cm down-slope of the manure application zone, with samples from the two collection times collected directly adjacent to each other. Field collection utensils were wiped clean between uses and sprayed with ethanol. Soil and manure samples ($n = 10$ manure and 40 soil) were placed in coolers and transported immediately on ice to the laboratory for analyses.

2.2 | Microbiological analyses

Within 4 h of collection, the soil and manure samples were homogenized by hand mixing and stored in a −80 °C freezer on arrival to the laboratory. The DNA was isolated from the 40 soil and 10 manure samples using Qiagen DNeasy Power Soil Kit (#12888-100) following manufacturer's instructions, except cell lysis was done with an Omnibeadruptor12 (Omni International), at 2.40 m s^{-1} for 1 min. Standard polymerase chain reaction (PCR) was used to assay 14 tetracycline resistance genes, sulfonamide *sul1*, macrolide *erm*(B), b-lactamase CTX-M-32, and integrase *intI*1 (Supplemental Figure S1). All PCR and quantitative PCR (qPCR) details are presented in Supplemental Table S2. The PCR assay reaction mix consisted of 1 × JumpStart REDTaq ReadyMix reaction mix (Sigma Chemical), 2.0 µM primers, PCR-grade water, and DNA template. Thermocycling conditions are presented in Supplemental Table S3. Amplicons were visualized on a 2% agarose gel (1 × TAE) stained with SYBR Safe DNA gel stain (LifeTech) and imaged using the UVP GelDoc-It^{Ts3} Imager (Analytik Jena US). Pre-application soil results were confirmed with additional PCR testing, including controls for PCR inhibition. Detection of the 16S ribosomal RNA (rRNA) gene was used to confirm that PCR inhibitors had been removed during soil DNA extraction from all samples.

Individual qPCR assays were used to quantify sulfonamide, erythromycin, b-lactamase, integrase, and 16S rRNA genes. All samples were run in triplicate. Thermocycling conditions are listed in Supplemental Table S4. Quality conditions consisted of an efficiency between 90 and 110% and an R^2 value between .80 and 1.00. Melting curve analysis was performed for all assays to confirm authenticity of the PCR product. Limits of detection for the assays were determined by running, in triplicate, a series of dilutions using the gBlock (IDT) positive control, choosing the lowest dilution that amplified all samples with a standard deviation of Cq values (i.e., how many cycles were required to detect a measurable signal) that was <1.0. The limits of detection (LODs) were four, three, four, and three copies per PCR reaction for *sulI, erm*(B), CTX-M-32, and *intI1*, respectively. On a per-gram-soil basis, the LODs would be 1200–1600 copies g^{-1} (0.25 g soil extracted, 100 µl purified DNA extract, 1 µl amplified in each reaction). Samples were assayed for an additional nine clinically relevant β-lactamases using qPCR: CMY-2, CTX-M-15, CTX-M-14, OXA-48, IMP, VIM, DHA, KPC, and NDM, using the ARM-D Kit (Streck) for β-lactamase following manufacturer's instructions.

It is common to normalize ARG qPCR field measurements using the 16S rRNA gene, and these values are commonly displayed as the negative log of the normalized values. Here, we expressed these values as the number of ARG targets per 100,000 16S rRNA genes, to align with incidence rate data commonly used to assess risk in public health settings.

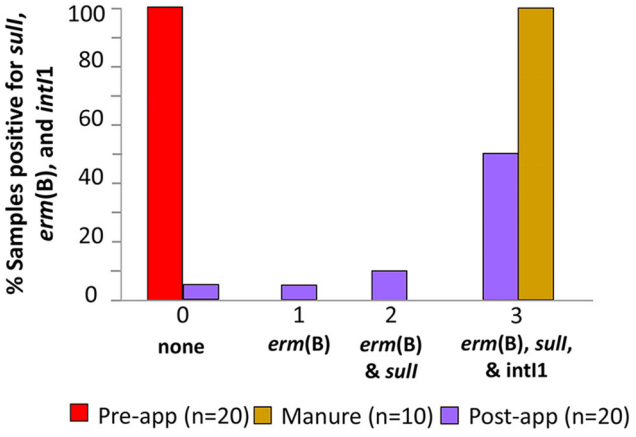

FIGURE 1 Percentage of 20 plots positive for individual tetracycline (TET) resistance genes. $N = 10$ manure. $N = 20$ post-application soils. Manure-borne antibiotic resistance genes (ARGs) account for many, but not all, of the ARGs enriched in soil after land application of swine slurry

3 | RESULTS

Antibiotic resistance gene detection varied, depending on the specific target measured, but all manure and soil samples were negative for the 10 β-lactamase genes surveyed and positive for 16S rRNA genes. Changes in soil antibiotic resistance gene profiles were observed for 17 out of 27 assayed genes after manure application and multiple rainfall events, with the remaining genes not detected in any samples.

3.1 | Qualitative or presence

Fourteen tetracycline resistance (TET^r) genes were assayed from the soil pre-application ($n = 20$), manure ($n = 10$), and soil post-application ($n = 20$) samples using standard PCR. None of the assayed TET^r genes were detected in pre-application soils, despite their presence in PCR positive controls. Out of the 14 TET^r genes assayed, 12 were detected in manure and post-application soils (Figure 1). All manure samples were positive for the same nine of the possible 14 TET^r genes. There was no change in the target ARG profile of the applied manure over the course of the experiment. The most frequently detected TET^r genes in the post-application soil were *tet*(L), *tet*(M), and *tet*(O), which were found in 56% of post-application soil samples. Each of the 20 post-application soils had between zero and nine TET^r genes detected of the 14 that were assayed (Figure 1). Neither *tet*(E) nor *tet*A(P) were detected in any of the post-application soils. The mean

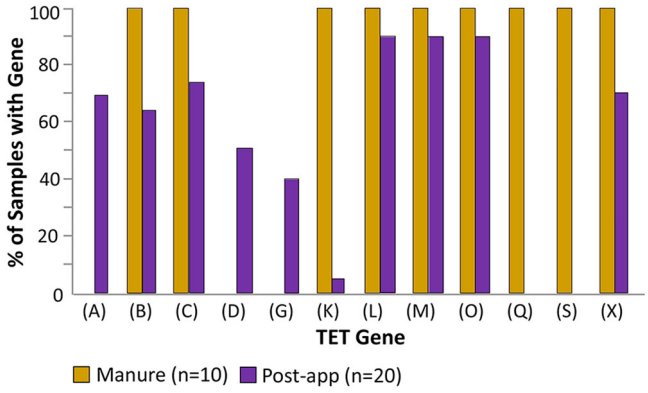

FIGURE 2 Carriage of *sulI*, *erm*(B), and *intI1* genes. Soil antibiotic resistance gene (ARG) profile changed after manure application and simulated rainfall

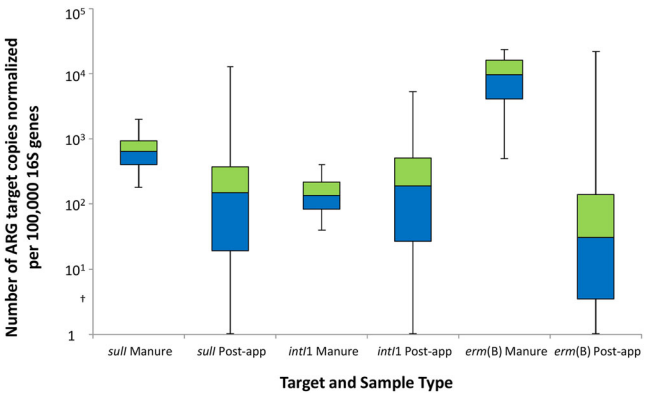

FIGURE 3 Quantification of antibiotic resistance gene (ARG) targets, normalized per 100,000 16S genes. Mean absolute values, although valuable for risk assessment purposes, obscure ecologically relevant information, such as variations in bacterial community, 16S copy number, and plot-based differences in ARG persistence. Lowest normalized copy number per 100,000 16S ribosomal RNA genes is zero

number of TET^r genes detected in manure and post-application soils were nine and six, respectively.

Standard PCR was also used to assay sulfonamide, macrolide, β-lactamase, and integrase genes (Figure 2). All samples were negative for β-lactamase (CTX-M-32) resistance genes. All manure samples were positive for sulfonamide resistance genes, macrolide resistance genes, and integrase. Of the post-application soil samples, 85% were positive for sulfonamide resistance genes, macrolide resistance genes, and integrase (17 of 20 post-application samples).

3.2 | qPCR (quantitative measures of antibiotic resistance)

The average number of copies of *sulI*, *erm*(B), CTX-M-32, *intI*1, and 16S rRNA in pre-application soil, manure, and post-application soil are presented in Table 1. Values were normalized based on the total number of 16S rRNA genes in the sample and are displayed in Figure 3. There were nine additional β-lactamase genes assayed using qPCR: CMY-2, CTX-M-15, CTX-M-14, OXA-48, IMP, VIM, DHA, KPC, and NDM. All soil and manure samples were negative for the nine additional β-lactamase genes.

4 | DISCUSSION

Methods that employ PCR are widely used in a research capacity for detecting antibiotic resistance in environmental samples (Marti et al., 2013: McKinney et al., 2018), offering the possibility to obtain information on target genes in hours as compared with culture-based methods that require days of laboratory work. However, PCR-based detection of ARGs from complex environmental samples also has limitations that make it difficult to compare results between studies or develop standardized environmental ARG monitoring efforts (Luby, Ibekwe, Ziles, & Pruden, 2016). The first step to determine the potential for causal links between land application of animal manures and antibiotic resistance-derived adverse human or veterinary health outcomes (Williams-Nguyen et al., 2016) is to accurately measure antibiotic resistance in soil samples.

4.1 | Quantifying antibiotic resistance genes for individual plots versus mean

The qPCR method was used to quantify *sulI*, *erm*(B), CTX-M-32, *intI*1, and 16S rRNA genes (Gillings et al., 2015; Marti et al., 2014; Pruden, Pei, Sorteboom, & Carlson, 2006) from 20 replicate field plots, at two time points, and then normalized using 16S rRNA gene abundance. Looking at the normalized individual plot values contributing to the mean for these targets (Figure 3), it is evident that there was a large range of values across plots. For example, *sul1* genes in post-application soil ranged from 0 to 12,400 copies. This highlights the heterogeneity associated with field measurements of ARGs. Mean values, although useful as a general summary tool, obscure the plot-level variability observed in this study. When collecting soils for field studies of manured soils, it is recommended to pool subsamples from multiple locations and homogenize thoroughly to minimize impact of soil heterogeneity on ARG conclusions.

The mean absolute values of the *sulI*, *erm*(B), and *intI1* ARGs per gram dry weight of soil in the post-application samples was higher than the combined mean values in the pre-application soil and applied manure (Table 1). This is likely due to the moist conditions after the rainfall events, in conjunction with an average Nebraskan July temperature of 32 °C—ideal conditions for manure- or soil-associated

TABLE 1 Quantitative polymerase chain reaction (qPCR) gene summary for sulfonamide *sul1*, erythromycin *erm*(b), β-lactamase (ctx-m-32), and an integrase gene (*inti*1). An average moisture level of 90% was used to calculate the dry matter for manure (https://extension2.missouri.edu/eq215)

Gene	Pre-application soil	Manure	Post-application soil
	copies g^{-1} soil DM	copies g^{-1} manure DM	copies g^{-1} soil DM
sul1	2.12×10^3	1.91×10^6	4.37×10^7
erm(B)	ND	2.29×10^7	9.57×10^8
CTX-M-32	ND	ND	ND
*intI*1	3.17×10^3	3.29×10^5	4.12×10^7
16S ribosomal RNA	1.28×10^9	5.19×10^8	7.06×10^9

Note. DM, dry mass; ND, not detected.

microbes to proliferate and metabolize in the soil. Marti et al. (2014) hypothesized that the cooler and wetter spring weather was responsible for observed bacterial blooms, compared with warmer, drier conditions under which an immediate exponential decrease in bacteria was observed. Based on the current study and the results of Marti et al. (2014), it appears that temperature was less important than moisture in supporting proliferation of manure-borne bacteria after land application.

4.2 | Standard PCR screening for tetracycline resistance genes

Tetracycline resistance genes (TET^r) are frequently assayed in agricultural soils and are generally considered common (Cytryn, 2013; D'Costa et al., 2006; Durso et al., 2012). In the current study, none of the assayed TET^r genes were detected in pre-application soil samples. This result was unexpected, as previous studies have identified TET^r genes in nonmanured agricultural soils, organic farm soils, and prairie soils (Agga, Arthur, Durso, Harhay, & Schmidt, 2015; Cadena et al., 2018; Durso, Wedin, Gilley, Miller, & Marx, 2016). Unlike pre-application samples, multiple TET^r genes were detected in both manure and post-application soil samples (Figure 1); these data highlight the utility of TET^r genes as a marker for manure inputs at this site and provide evidence that applied manure is the primary source of the TET^r genes found in the post-application soils in this study.

Of note, however, is that three TET^r genes [*tet*(A), *tet*(D), and *tet*(G)] detected in post-application soils were absent in both pre-application soils and the source manure. We have two possible explanations:

1. Results could be due to soil heterogeneity. Post-application samples were collected directly adjacent to pre-application soils, but the destructive nature of field collection means the two samples were not identical.
2. It is possible that the genes were present in either the pre-application soils or source manure, but at a level below our detection limit of three gene copies per PCR reaction (equivalent to 1.2×10^3 copies per gram of soil), and then the genes were enriched after the rainfall events.

Antibiotic resistance genes are widely considered to have originated in environmental bacteria (Perry, Waglechner, & Wright, 2016), and environmental bacteria remain an important reservoir for ARGs. (Gibson, Forsberg, & Dantas, 2015). Antibiotic resistance genes, including TET^r genes, are also common in feces, including feces of cattle, swine, humans, dogs, and fish (Brooks, Adeli, & McLaughlin, 2014; Durso et al., 2012). Tetracycline resistance genes evolved long before the discovery of antibiotics (Koike, Mackie, & Aminov, 2017; Rahman, Sakamoto, Kitamura, Nonaka, & Suzuki, 2015). However, the use of tetracycline, along with other selective pressures, has resulted in an increased proportion of bacteria harboring ARGs in clinical and environmental samples (Tan et al., 2018). This includes TET^r genes found in both organic and conventional farming systems (Brooks et al., 2014; Cadena et al., 2018; Marti et al., 2013, 2014). In this study, using plots that had never received any manure samples, the pre-application soils were negative for the TET^r genes assayed. In the current set of experiments, the swine manure harbored a distinct set of TET^r genes compared with the receiving agronomic soil.

For research and surveillance efforts, the specific ARG target measured has been shown to affect the conclusions of the study (Cadena et al., 2018; Durso et al., 2016; Liu, Jia, He, Zhang, & Ye, 2017; Walk et al., 2007). Even when associated with the same drug resistance category such as tetracycline, individual ARG targets, and sometimes even specific gene subtypes are not equivalent (Rahman et al., 2015). In the current study, we observed a smaller number of agronomic plots positive for all TET^r genes measured, compared with amounts originating from the source manure; however, the decrease varied considerably depending on the individual TET^r gene assayed (range 0–90%) (Figure 1). Contributing to this phenomenon is the fact that the types of genes detected in a sample depend, to a large extent, on the types of bacteria present (Durso et al., 2012; Durso, Miller, Schmidt, &

Callaway, 2017; Forsberg et al., 2014; Liu et al., 2017). This is particularly well documented for TET^r genes (Roberts & Schwarz, 2016; https://faculty.washington.edu/marilynr/).

The practical implications of individual TET^r genes being nonrandomly distributed in the environment is that target selection can influence the perceived efficacy of control measures, with different TET^r genes allowing for separate conclusions. For example, the short land application period for the current work resulted in manure-borne *tet*(Q) and *tet*(S) being nondetectable just days after manure application, but *tet*(L), *tet*(M), and *tet*(O) remained detectable in 90% of the plots. Thus, multiple TET^r gene targets need to be used to account for the differing persistence of individual ARGs. Furthermore, the background ARG profile of individual field sites must be considered (Durso et al., 2016).

Manure is an essential element of sustainable organic cropping systems, and responsible manure management is key to minimizing numerous adverse environmental and human health impacts. The addition of ARGs to soil, via manure application, results in an immediate increase in the number and types of soil ARG, due to a simple additive effect (Durso & Cook, 2014). There is also evidence that manure application enriches soil-borne bacteria, including indigenous bacteria that carry ARGs (Udikovic-Kolic, Wichmann, Broderick, & Handelsman, 2014), and that manure-borne bacteria can persist (Scott et al., 2018) and replicate in the environment (Walk, Alm, Calhoun, Mladonicky, & Whittam, 2007). Another important consideration in the study of ARG presence in manure-amended soils is high variability. Although the results from the current study demonstrated averaged potential increases in ARGs after land application of swine manure and rainfall, there was variability observed among the 20 replicate plots. These observations are supported by the work of Marti et al. (2014) and Udikovic-Kolic et al. (2014). However, it would be presumptuous to draw any broad conclusions on heterogeneity of ARG presence within plots based on these limited data. There were two data points that were significant outliers (Figure 3), potentially due to uneven distribution of the manure. Variability was also observed in the quantity of specific gene targets, supporting the idea that individual ARG targets are not equivalent for measuring general antibiotic resistance (Durso et al., 2016).

Data from this study highlight the challenge of differentiating the true effect of manure application from ARG background noise under field conditions when measuring frequency of ARG occurrence and abundance, even when there is a high degree of replication within the study. Our results support recommendations to collect background or baseline data, particularly when examining impacts of human or animal activities on ARG, so that results can be interpreted in light of the amounts and kinds of targets already present before the treatment or application began (Dungan, McKinney, & Leytem, 2018; Durso & Cook, 2014; Rothrock et al., 2016).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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SUPPLEMENTAL MATERIAL

Meyers et al. Antibiotic resistant gene profile changes in cropland soil following manure application and rainfall. J. Environ. Qual.

Supplemental Table S1. Soil and Site Description. From Gilley et al. (2017)

Characteristic or Descriptor	Value
Farm size	320 Acres
Cropping Management	Long-term no-till, with controlled wheel traffic
Crops and rotations	Winter wheat, soybeans, corn, grain sorghum.
Crop immediately before study	Winter wheat
Weed control year before study	Glyphosate
Residue status during experiment	Wheat residue not chopped or removed
Percent of soil coverage from residue	100%
Crop residue present at time of field test	7.73 Mg ha-1
Development of site	Loess under prairie vegetation
Soil particle size	fine, smectitic, mesic Typic Argiudoll
Sand:silt:clay ratios	22% sand, 44% silt, and 34% clay
Saturated hydraulic conductivity	Moderately low
Hydrologic soil group	C
Mean Bray and Kurtz No. 1 phosphorus	17.9mg kg^{-1}
Water soluble phosphorus	1.7 mg kg^{-1}
Nitrogen as NO_3	9.4 mg kg^{-1}
Mean slope gradient	4.9%
Electrical conductivity	0.51 dS m^{-1}
pH	6.7
Organic matter	38 kg^{-1}
Total carbon content	22 g kg^{-1}
Boron	2.07 mg kg^{-1}
Calcium	3834 mg kg^{-1}
Chloride	2.81 mg kg^{-1}
Magnesium	508 mg kg^{-1}
Magnesium	12.7 mg kg^{-1}
Potassium	393 mg kg^{-1}
Sodium	24.9 mg kg^{-1}
Sulfate	71.9 mg kg^{-1}

Supplemental Table S2: Primer information for PCR and qPCR for antibiotic resistance genes

Primer Name	Sequence	Amplicon Size (bp)	Annealing Temp (ºC)	Notes
Ng *tet*(A) F	5'-GCT ACA TCC TGC TTG CCT TC-3'	210	58	Efflux
Ng *tet*(A) R	5'-CAT AGA TCG CCG TGA AGA GG-3'			
Ng *tet*(B) F	5'-TTG GTT AGG GGC AAG TTT TG-3'	659	58	Efflux
Ng *tet*(B) R	5'-GTA ATG GGC CAA TAA CAC CG-3'			
Ng *tet*(C) F	5'-CTT GAG AGC CTT CAA CCC AG-3'	418	58	Efflux
Ng *tet*(C) R	5'-ATG GTC GTC ATC TAC CTG CC-3'			
Ng *tet*(D) F	5'-AAA CCA TTA CGG CAT TCT GC-3'	787	58	Efflux
Ng *tet*(D) R	5'-GAC CGG ATA CAC CAT CCA TC-3'			
Ng *tet*(E) F	5′-AAA CCA CAT CCT CCA TAC GC-3′	278	58	Efflux
Ng *tet*(E) R	5′-AAA TAG GCC ACA ACC GTC AG-3′			
Ng *tet*(G) F	5′-CAG CTT TCG GAT TCT TAC GG-3′	844	58	Efflux
Ng *tet*(G) R	5′-GAT TGG TGA GGC TCG TTA GC-3′			
Ng *tet*(K) F	5′-TCG ATA GGA ACA GCA GTA-3′	169	50	Efflux
Ng *tet*(K) R	5′-CAG CAG ATC CTA CTC CTT-3′			
Ng *tet*(L) F	5′-TCG TTA GCG TGC TGT CAT TC-3′	267	57	Efflux
Ng *tet*(L) R	5′-GTA TCC CAC CAA TGT AGC CG-3′			
Ng *tet*(M) F	5′-GTG GAC AAA GGT ACA ACG AG-3′	406	57	Ribosomal
Ng *tet*(M) R	5′-CGG TAA AGT TCG TCA CAC AC-3′			
Ng *tet*(O) F	5′-AAC TTA GGC ATT CTG GCT CAC-3′	515	57	Ribosomal
Ng *tet*(O) R	5′-TCC CAC TGT TCC ATA TCG TCA-3′			
Ng *tet*(A)P F	5′-CTT GGA TTG CGG AAG AAG AG-3′	676	58	Efflux
Ng *tet*(A)P R	5′-ATA TGC CCA TTT AAC CAC GC-3′			
Ng *tet*(Q) F	5′-TTA TAC TTC CTC CGG CAT CG-3′	904	58	Ribosomal
Ng *tet*(Q) R	5′-ATC GGT TCG AGA ATG TCC AC-3′			
Ng *tet*(S) F	5′-CAT AGA CAA GCC GTT GAC C-3′	667	58	Ribosomal
Ng *tet*(S) R	5′-ATG TTT TTG GAA CGC CAG AG-3′			
Ng *tet*(X) F	5′-CAA TAA TTG GTG GTG GAC CC-3′	468	58	Enzymatic
Ng *tet*(X) R	5′-TTC TTA CCT TGG ACA TCC CG-3′			
Pei *sulI* F	5'-GAC GAG ATT GTG CGG TTC TT-3'	185	64	Enzymatic
Pei *sulI* R	5'-GAG ACC AAT AGC GGA AGC C-3'			
Chen *erm*(B) F [PCR]	5′-GATACCGTTTACGAAATTGG-3′	364	58	Efflux
Chen *erm*(B) R	5′-GAATCGAGACTTGAGTGTGC-3′			
Florez *erm*(B) F [qPCR]	5′-GGATTCTACAAGCGTACCTTGGA-3′	69	60	Efflux
Florez *erm*(B) R	5′-AATCGAGACTTGAGTGTGCAAGAG-3′			
Bellanger 16S rRNA F	5′-CCTACGGGAGGCAGCAG-3′	195	55	rRNA
Bellanger 16S rRNA R	5′-ATTACCGCGGCTGCTGG-3′			
Hardwick *intI1* F [PCR]	5′-CTGGATTTCGATCACGGCACG-3′	473	60	Integrase
Hardwick *intI1* R	5′-ACATGCGTGTAAATCATCGTCG-3′			
Barraud *intI1* F [qPCR]	5′-GATCGGTCGAATGCGTGT-3′	196	55	Integrase
Barraud *intI1* R	5′-GCCTTGATGTTACCCGAGAG-3′			
Szczepanowski *ctx-m32* F	5′-CGTCACGCTGTTGTTAGGAA-3′	185	63	β-lactamase
Szczepanowski *ctx-m32* R	5′-CGCTCATCAGCACGATAAAG-3′			

Supplemental Table S3: Conditions for PCR thermocycling of individual antibiotic resistance genes

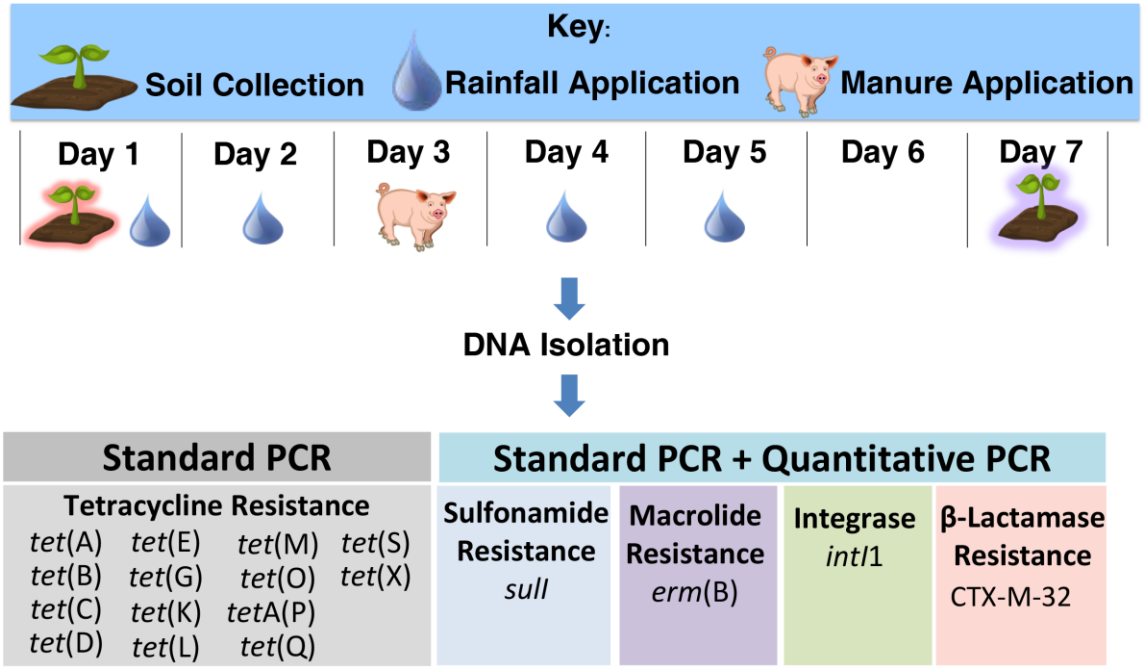
Gene	PCR Thermocycling Conditions Used	References
Tetracycline *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*A(P), *tet*(Q), *tet*(S), *tet*(X)	**1 cycle of:** 94°C for 2 min **30 cycles of:** 94°C for 30 sec, annealing temp* for 30 sec, 72°C for 1 min **1 cycle of:** 72°C for 5 minutes.	Ng et al., 2001
Sulfonamide *sulI*	**1 cycle of:** 94°C for 2 min **35 cycles of:** 94°C for 30 sec, annealing temp* for 30 sec, 72°C for 2 min **1 cycle of:** 72°C for 5 minutes.	Pei et al., 2006
Erythromycin *erm*(B)	**1 cycle of:** 94°C for 2 min **35 cycles of:** 94°C for 30 sec, annealing temp* for 30 sec, 72°C for 2 min **1 cycle of:** 72°C for 5 minutes.	Chen et al., 2007
Integrase *intI1*	**1 cycle of:** 94°C for 2 min **35 cycles of:** 94°C for 30 sec, annealing temp* for 30 sec, 72°C for 2 min **1 cycle of:** 72°C for 5 minutes.	Hardwick et al., 2008
β-lactamase *ctx-m32*	**1 cycle of:** 94°C for 2 min **35 cycles of:** 94°C for 30 sec, annealing temp* for 30 sec, 72°C for 2 min **1 cycle of:** 72°C for 5 minutes.	Szczepanowski et al., 2009
16S rRNA	**1 cycle of:** 95°C for 15 min **35 cycles of:** 95°C for 15 sec annealing temp* for 20 sec, 72°C for 10 sec.	Bellanger et al, 2014

*Annealing temperatures are presented in Table S2

Supplemental Table S4: Conditions for qPCR thermocycling of antibiotic resistance genes

Gene	qPCR Thermocycling Conditions Used	Reference
Sulfonamide *sulI*	1 cycle of 95°C for 15 min; 40 cycles of 95°C for 15 sec, 65°C for 30 sec and 72°C for 30 sec; and a melt curve from 60°C-95°C.	Pei et al., 2006
Erythromycin *erm*(B)	1 cycle of 95°C for 15 min; 40 cycles of 95°C for 15 sec, 60°C for 60 sec; and a melt curve from 60°C-95°C.	Florez et al., 2014
Integrase *intI1*	1 cycle of 95°C for 15 min; 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 10 sec; and a melt curve from 60°C-95°C.	Barraud et al., 2010.
β-lactamase *ctx-m32*	1 cycle of 95°C for 15 min; 40 cycles of 95°C for 15 sec, 63°C for 30 sec and 72°C for 10 sec; and a melt curve from 60°C-95°C.	Szczepanowski et al., 2009
16S rRNA	1 cycle of 95°C for 15 min; 35 cycles of 95°C for 15 sec, 55°C for 20 sec and 72°C for 10 sec; and a melt curve from 60°C-95°C.	Bellanger et al., 2014

Supplemental Figure S1: Experimental design for study of presence of antibiotic resistance genes in soils after swine slurry application and simulated rainfall



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