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Fate of antimicrobials and antimicrobial resistance genes in simulated swine manure storage

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HIGHLIGHTS

- Decay rates were determined for antimicrobials in anaerobic swine manure slurry.
- Decreases in *tet* and *erm* resistance genes were observed.
- Reductions in *tet* genes corresponded with reduced concentrations of chlortetracycline.
- Compounds in addition to parent antimicrobial may exert selective pressure for *erm* resistance.

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ABSTRACT

The behavior of three antibiotics (bacitracin, chlortetracycline, and tylosin) and two classes of antibiotic resistance genes (ARGs), *tet* and *erm*, were monitored in swine manure slurry under anaerobic conditions. First-order decay rates were determined for each antibiotic with half-lives ranging from 1 day (chlortetracycline) to 10 days (tylosin). ARGs were monitored in the swine manure slurry, and losses of approximately 1 to 3 orders of magnitude in relative abundance were observed during the 40 day storage period. First-order degradation profiles were observed for chlortetracycline and its corresponding resistance genes, *tet*(X) and *tet*(Q). Tylosin was degraded to approximately 10% of the starting concentration by day 40; however, the relative abundance of *erm*(B) remained at 50–60% of the initial relative abundance while the relative abundance of *erm*(F) decreased by 80–90%, consistent with tylosin. These results indicate that *tet* resistance genes respond primarily to chlortetracycline antimicrobials, and may be lost when the parent tetracycline compound is degraded. In contrast, *erm*(B) resistance gene may respond to a range of antimicrobials in animal manure, and may persist despite losses of tylosin.

1. Introduction

Antimicrobial resistance is among the world's most pressing public health concerns and the presence of both antimicrobial resistant bacteria and mobile antimicrobial resistance genes (ARGs) in the environment contribute to the evolution and spread of antibiotic resistance (Wellington et al., 2013). Wastes generated from animal production represent a major source of antimicrobials and ARGs to the environment (Pruden et al., 2013; Wang et al., 2012).

Antimicrobials are used in animal production at subtherapeutic levels for growth promotion and prophylaxis and at therapeutic levels for disease treatment. Antimicrobials added to animal feed are not completely absorbed during digestion, resulting in their presence in manure (Heuer et al., 2011). The presence and activity of antimicrobials in manure can select for antimicrobial resistant bacteria, even at low antimicrobial concentrations (Knapp et al., 2008) and antimicrobials, ARGs, and resistant bacteria can enter the environment through a variety of pathways including agricultural wastewater (Wantanabe et al., 2010; Zhang et al., 2013) or land applied animal manure (Zilles et al., 2005; Pei et al., 2006; Heuer et al., 2011; Joy et al., 2013).

Swine production in the United States was nearly 117 million heads in 2012 (USDA, 2013), and each animal can produce approximately 1500 kg of fresh manure by the time they reach market weight (Richert et al., 2005). Bacitracin A, chlortetracycline, and tylosin are antimicrobials commonly used in swine production (Cromwell, 2002; Jindal et al., 2006) and antimicrobial excretion rates of up to 90% in urine and 75% in feces have been reported (Halling-Sørensen et al., 2001). Swine produced at confined animal feeding operations (CAFOs) typically use one of three waste handling systems: flush systems, pit

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recharge, or deep pits (Sarmah et al., 2006). In deep pit systems, manure falls from a slatted floor into a pit below the animal housing facility and typically uses less water than either flush or pit recharge systems (Sarmah et al., 2006). Manure may be stored in these pits for up to a year. Deep pit systems are commonly used in colder climates such as the upper Midwest in the United States and manure accumulating in deep pits provides an environment for anaerobic microbial activities.

Relatively little information is available regarding the concurrent fate of antimicrobials and ARGs during anaerobic swine manure storage. Few studies have evaluated the fate of antimicrobials or ARGs in swine waste lagoons or storage pits. Evidence suggests that degradation of parent antimicrobials may occur during storage, but may not result in concurrent decreases in ARGs. Stone et al. (2009) found a 57% reduction in chlortetracycline concentrations and a 100% reduction in tylosin concentrations over 216 days in laboratory scale anaerobic batch experiments, where initial concentrations of chlortetracycline and tylosin were 28 and 1.1 mg/L, respectively. However, Chen and co-workers concluded that mesophilic anaerobic digestion and lagoon storage could not effectively reduce the absolute abundance of tetracycline and erythromycin resistance genes (Chen et al., 2010; Wang et al., 2012). Oxygen may also affect the fate of ARGs during swine manure storage. Diehl and LaPara (2010) tested the effects of oxygen and temperature on the degradation of ARGs in the biosolids of a wastewater treatment plant. They observed deceases in ARGs in anaerobic digesters under high temperature, while detecting no evident ARG decrease in aerobic digesters at the temperatures that were tested. Another study reported increases in ARGs during manure storage under aerobic conditions (Heuer et al., 2008). In that study, sulfonamide resistance genes *sul*(I) and *sul*(II) increased exponentially during the first 60 days of storage. Persistence of both antimicrobials and ARG in livestock manure determines subsequent entry into the environment through land application and the resulting potential for transport from agricultural watersheds.

The objectives of this study were to quantify the concentrations of three antimicrobials commonly used in swine production: bacitracin A, chlortetracycline, and tylosin, and their corresponding ARGs over time under simulated deep pit swine manure storage and to determine if the loss of the parent antimicrobial corresponds to decreased levels of antibiotic resistance genes in manure.

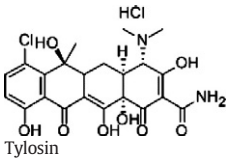
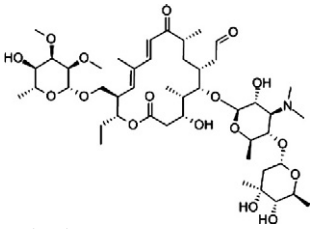
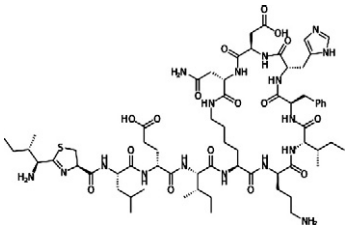
2. Methods and materials

2.1. Laboratory manure storage experiments

Swine manure was collected from the U.S. Meat Animal Research Center near Clay Center, Nebraska from separate barns where animals were administered bacitracin A, chlortetracycline, or tylosin. The chemical structure and physical–chemical properties of the antimicrobials can be found in Table 1. All animals were fed a corn and soybean-based diet with controlled dosages of antimicrobials and other supplements. Replacement gilts were fed 39.7 mg bacitracin A per kg feed, feeder pigs were fed 110.2 mg chlortetracycline per kg feed, and sows and gilts were fed 114.6 mg tylosin per kg feed. Fresh manure was collected directly from the floor in each animal housing unit utilizing one of the three target antimicrobials and transported to Lincoln, Nebraska where it was placed in experimental reactors. Additional properties of manure collected from the same facility are provided in Table 2.

100 mL glass amber wide mouth jars were used as sacrificial reactors. Manure and water were mixed in a 2:1 (w/w) ratio, and the homogenized mixture was allocated to each reactor for a total mass of 75 g (Masse et al., 2000). Reactors were sparged with nitrogen in an anaerobic chamber for approximately 5 min and incubated at 37 °C for up to 40 days. The reactor caps were briefly loosened every 1–2 days to prevent methane buildup within the reactors. Duplicate reactors were sacrificed at pre-determined periods. Samples were frozen at −20 °C until antimicrobial and ARG analyses were performed.

Table 1
Parent antimicrobial chemical structure and selected physical–chemical properties.[1]

Chlortetracycline 	Log K_{ow} = −0.62 K_d = 501–3715 L/kg (Teixidó et al., 2012) Solubility = 500 mg/L
Tylosin 	Log K_{ow} = 3.5 K_d = 1300 L/kg (Clay et al., 2005) Solubility = 6000 mg/L
Bacitracin A 	Estimated Log K_{ow} = −3.3 Estimated solubility = 1.5 μg/L

[1] Estimated properties are from US EPA EpiSuite Program v. 4.11.

2.2. Antimicrobial analysis

Antimicrobials were extracted from manure using solvent extraction followed by solid phase extraction (SPE) cleanup. Approximately 0.2 g of sample was mixed with 5 g of clean sand and spiked with 16 ng oleandomycin as a surrogate to monitor analyte recovery, followed by the addition of 14 mL of 5 mM ammonium citrate (buffered to pH 6 using ammonium hydroxide) and 6 mL methanol in 50-mL polypropylene centrifuge tubes. Mixtures were shaken briefly by hand and then on a Burrell wrist-action shaker for 30 min. Solids were extracted a second time with 4 mL of ammonium citrate and 16 mL methanol, and a third time using 20 mL acetone. All extracts were combined and fortified with internal standards (doxycycline and roxithromycin, 40 ng each) and then concentrated on a Labconco RapidVap N_2 sample concentrator (Labconco Corporation, Kansas City, MO) at 30 °C (90% rotation speed) until the volume was reduced by half. Purified reagent water was added to bring the extract volume to 100 mL and the resulting aqueous solutions were extracted using 200 mg Oasis HLB SPE cartridges. SPE cartridges were eluted into borosilicate test tubes using 3 mL of 130 mM ammonium citrate in methanol. The solvent was reduced in volume to approximately 200 μL under a stream of dry nitrogen, and transferred to an autosampler vial with silane-treated insert and then mixed with 200 μL reagent water. Roxithromycin was used as an internal standard for tylosin and bacitracin, while doxycycline was used for chlortetracycline. Recovery of bacitracin A, chlortetracycline, and tylosin, was determined from extraction and analysis of fortified reagent water during the elution stage. Fortified blanks and method blanks were analyzed at a frequency of 1 in 20 samples (5%). Method detection limits were determined by extraction and analysis of 8 replicates of reagent water samples fortified with antimicrobials at 0.005 μg/L. Method detection limits determine by extraction and analysis of 8 replicates of manure solids ranged from 0.5 ng/g for tylosin and chlorotetracycline, to 3 ng/g for bacitracin A and F. Recovery of target antibiotics as quantified by recovery of the surrogate were 95 ± 22% for chlortetracycline, 114 ± 29% for bacitracin, and 140 ± 68% for tylosin.