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Trang Thi Thu Hoang
Iowa State University

Michelle L. Soupir
Iowa State University, msoupir@iastate.edu

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Abstract

Tylosin is an antibiotic commonly used in swine industry at subtherapeutic levels to improve growth rates and efficiency of feed utilization. When manure is applied to subsurface drained agricultural fields, antibiotic residues in manure provide selective pressure for the development of microbial resistance. Transport of these microorganisms through soils into tile drainage lines and ultimately into surface waters is a serious threat for public health. This study was performed to investigate the occurrence and transport of tylosin-resistant enterococci from tile drained agricultural fields receiving semi-annual swine waste applications. The field study was conducted at the Iowa State University Northeast research farm at Nashua, Iowa in April and November of 2009. Liquid swine slurry from an operation feeding tylosin at subtherapeutic levels was injected into no-till field plots. Samples collected from field were assayed for total enterococci concentration and enterococci exhibiting resistance to tylosin at an MIC of 35 mg/L. All the enterococci in manure samples were found to be resistant to tylosin. Concentrations of total and tylosin resistant enterococci in soil samples was 6.84×10^5 cfu/100ml and 5.25×10^5 cfu/100ml, respectively. There was a statistically significant difference between the total and tylosin-resistant enterococci concentrations in water samples ($p=0.05$). Total enterococci concentrations in water samples ranged from 1.3×10^1 to 5.0×10^3 cfu/100mL while tylosin resistant enterococci concentrations ranged from 1.3×10^1 to 1.2×10^3 cfu/100mL. The percent of tylosin resistant enterococci were highest in manure samples and lowest in drainage water samples, suggesting that enterococci lose resistance as selective pressure from antimicrobial residues decrease.

Keywords

Enterococci, Tylosin-resistant enterococci, Microbial transport, Swine waste, Antibiotics

Disciplines

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Trang T. Hoang

Agricultural and Biosystems Engineering, Iowa State University, 3145 NSRIC Building, Ames, IA 50011, United States; tranghtt@iastate.edu

Michelle L. Soupir

Agricultural and Biosystems Engineering, Iowa State University, 3145 NSRIC Building, Ames, IA 50011, United States; msoupir@iastate.edu

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Abstract. Tylosin is an antibiotic commonly used in swine industry at subtherapeutic levels to improve growth rates and efficiency of feed utilization. When manure is applied to subsurface drained agricultural fields, antibiotic residues in manure provide selective pressure for the development of microbial resistance. Transport of these microorganisms through soils into tile drainage lines and ultimately into surface waters is a serious threat for public health. This study was performed to investigate the occurrence and transport of tylosin-resistant enterococci from tile drained agricultural fields receiving semi-annual swine waste applications. The field study was conducted at the Iowa State University Northeast research farm at Nashua, Iowa in April and November of 2009. Liquid swine slurry from an operation feeding tylosin at subtherapeutic levels was injected into no-till field plots. Samples collected from field were assayed for total enterococci concentration and enterococci exhibiting resistance to tylosin at an MIC of 35 mg/L. All the enterococci in manure samples were found to be resistant to tylosin. Concentrations of total and tylosin resistant enterococci in soil samples was 6.84×10^5 cfu/100ml and 5.25×10^5 cfu/100ml, respectively. There was a statistically significant difference between the total and tylosin-resistant

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enterococci concentrations in water samples ($p \leq 0.05$). Total enterococci concentrations in water samples ranged from 1.3×10^1 to 5.0×10^3 cfu/100mL while tylosin resistant enterococci concentrations ranged from 1.3×10^1 to 1.2×10^3 cfu/100mL. The percent of tylosin resistant enterococci were highest in manure samples and lowest in drainage water samples, suggesting that enterococci lose resistance as selective pressure from antimicrobial residues decrease.

Keywords. Enterococci, Tylosin-resistant enterococci, Microbial transport, Swine waste, Antibiotics.

Introduction

Iowa is one of the most heavily drained regions in the United States, impacting hydrology and contributing sediment and nutrients to aquatic systems; however, subsurface drainage tiles are an important part of farming systems necessary to improve yields in poorly drained soils. Iowa also leads the U.S. in swine production with more than 17 million hogs (USDA, 2006). Antibiotics such as tylosin are widely administered at sub-therapeutic levels by the swine industry to improve the growth rate and efficiency of feed utilization (Cromwell, 2002). When antibiotics are administered, only a fraction of the chemicals are utilized by the animals. The non-metabolized antibiotics or residues may remain unchanged through the animal digestion system and they are excreted in animal waste (Gustafson and Bowen, 1997; Onan and LaPara, 2003). Studies have demonstrated that up to 90% of administered antibiotics are released with urine and feces (Chander et al., 2006; Dolliver and Gupta, 2008).

Swine waste is often disposed of through land application, serving as a major source of nutrients and organics for soils and crops; however, the land application of livestock manure also introduces large amounts of antibiotics into the environment. *Tylosin* is among the most widely used of the macrolide class of veterinary antibiotics that targets the 50S ribosomal subunit, inhibiting transcription and eventually leading to cell death. Tylosin and its metabolites are typically excreted in manure: Kumar et al. (2004) reported tylosin concentrations in swine manure ranging from 0 to nearly 4 mg/L. Through land application, antibiotics may enter the soil and be transported into water systems by several different pathways (Kümmerer, 2009). Antibiotics residues in manure have been found to reach surface and ground waters (Davis et al., 2006; Pei et al., 2006) and ultimately could contribute to the development and spread of antibiotic resistance in the environment (Dolliver and Gupta, 2008) via intrinsic mutation or horizontal gene transfer. Many recent studies have found that numerous bacterial pathogens have become resistance to antibiotics (Heuer and Smalla, 2007; M. Kólar, 2002; Portillo et al., 2000).

Enterococci is one of the most common bacterial indicators of fecal pollution in water systems in the United States. They are one of the leading causes of nosocomial bacteremia, surgical wound infections, and urinary tract infections and are commonly found in the feces of humans and other warm-blooded animals. According to data from National Nosocomial Infections Surveillance system (NNIS system), 12% of nosocomial infection in the US from 1986 to 1989 were caused by enterococci (Emori and Gaynes, 1993) and recently enterococci have been developing resistance to many and sometimes all standard therapies. Recent studies reported the occurrence of enterococci in swine feces and waters near feeding operations or in runoff from land receiving animal waste applications (Sapkota et al., 2007; Soupir et al., 2006). Cools (2001) found that enterococci can survive up to 54 days at 25°C and 80 days at moisture contents of 100% field capacity.

Antibiotic resistant bacteria from manure can move to soils and water via infiltration (Chander et al., 2006; Storteboom et al., 2007). The physical processes controlling microbial movement through porous media are convection or advection or hydrodynamic dispersion; however, transport via macropores is considered to be one of the main pathways for microbial movement into subsurface waters (Beven and Germann, 1982). Although the occurrence of antibiotic-resistant bacteria near swine operations has been widely reported (Boxall et al., 2002; Campagnolo et al., 2002; Chenier and Juteau, 2009; Graham et al., 2009; Jindal et al., 2006; Koike et al., 2007), relationships between the source and the resistant microorganism in the stream are typically inferred due to farm and sample location instead of a clearly established hydrologic link. Understanding of

the release and transport of antibiotic-resistant bacteria from tile-drained fields receiving swine-waste application is limited. If resistant microorganism are transported through macropores and into tile lines, the tile lines will quickly facilitate the transport of these organisms to surface waters. The presence of antibiotic resistant bacteria in surface waters is a critical threat to human health due to increased likelihood of exposure through swimming and recreation activities. The goals of this study were to detect and quantify the occurrence of tylosin-resistant enterococci in (1) manure from farms feeding at sub-therapeutic levels; (2) soils amended with swine waste; and (3) tile drain flow from swine waste amended agricultural fields. This study will further our understanding about the occurrence and transport of antibiotic-resistant bacteria through tile drained lands from agricultural fields receiving swine waste. The results of this study will aid in assessment of the impacts of tile drainage management on the movement of microorganisms into surface waters and the impacts of antibiotic use in swine production on the aquatic environment and potentially human health.

Materials and Methods

To meet the stated objectives, a combination of field experiments and laboratory analysis was conducted. Liquid swine manure was collected from a farm administering tylosin at sub-therapeutic levels and applied to a subsurface drained plot. A rainfall simulator was used to apply rain to the plots until flow resulted in the tile drains. Water samples were collected at the outlet of the tile drained plot. Swine waste, soil, and water samples were analyzed for total enterococci and tylosin-resistant enterococci concentrations by membrane filtration.

Field studies Field experiments were conducted at Iowa State University's Experiment Station Research Farm near Nashua, Iowa. The site has a total of 36 plots 67m long by 56.7m wide; however, only two field plots were used in our experiments: plot 25 and plot 20 in experiments conducted in April and November of 2009, respectively. Soils at the site are poorly-drained and all plots have been under no-till management since 1979. Each plot is drained separately and has subsurface drainage lines installed in the center of the plot at a depth of 1.2 m below the ground surface with a drain spacing of 28.5 m. The central subsurface drainage lines are intercepted at the end of the plots and are connected to individual sumps for measuring drainage effluents and collecting water samples for analysis (Kanwar, 2006).

Swine waste from a nearby finishing facility which administers tylosin at a rate of 40 g/ton of feed was applied to an area of 929.03 m² within each plot and a sample was collected. Waste was injected into the no-till plots as shown in Figure 2. Soil samples were taken from the plot before and after manure application. A composite soil sample for the plot was created by mixing three replicate soil cores of 10 cm in depth at any three locations within each plot.

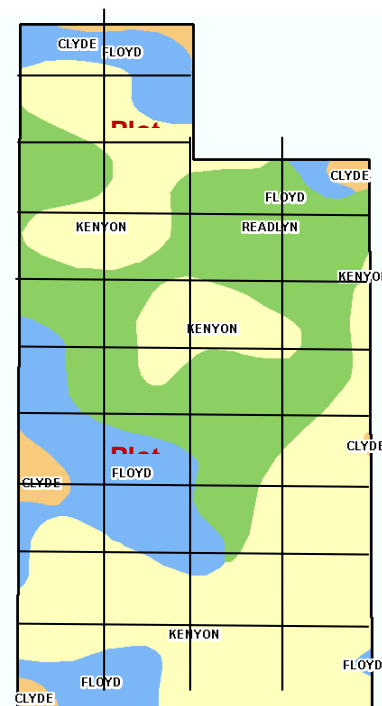


Figure 1. Soil types and locations of the two experimental plots at Iowa State University's Experiment Station Research Farm, Nashua, Iowa.

Water was applied to the soil surface using a boom, linearly moving rainfall simulator with an average intensity of two inches per hour. The rainfall simulator continued to move back and forth over the plot until flow commenced at the outlet of the tile drain. During the April 2009 simulation, water sampling began at the onset of flow in the tile drain, 53 minutes after the rainfall simulation started. During the November 2009 simulation, base-flow was present and therefore water sampling was initiated at the beginning of the rainfall simulation. Grab drain flow samples were collected every 4 to 7 minutes in the first hour after the start of flow and stored in sterile plastic 150 mL bottles (Figure 2). All samples were stored in a cooler before they were transported to the laboratory for analysis.



Manure sampling



Soil sampling



Water sampling

Figure 2. Sampling procedure

Analysis Manure, soil, and water samples were assayed for enterococci and enterococci exhibiting resistance to tylosin by membrane filtration (U.S. EPA, 1986). Soil and manure samples were diluted by phosphate buffered water prior to filtration. Concentration of total enterococci and tylosin-resistant enterococci were determined by enumerating colony forming units present on mEnt agar (U.S. EPA, 2000) without tylosin (control to account for the total enterococci population) and infused with tylosin (at the MIC level of 35 mg/L to account for tylosin-resistant enterococci) (Kaukas et al., 1988). Tylosin tartrate purchased from Sigma-Aldrich was used to prepare mEnt media with tylosin. A tylosin stock solution with concentration of 1000 mg/L was prepared and added to the agar when it cooled to a temperature of 50°C. The colony density per 100 mL was determined by the following equation:

$$\text{Enterococci}/100\text{mL} = \frac{\text{No. of enterococci colonies counted}}{\text{Volume of sample filtered (mL)}} \cdot 100$$

The presence of enterococci in the samples *was confirmed following EPA method 1600 and* the verification procedure is summarized in Table 1. Water samples were also analyzed for total suspended solids (TSS) by filtering samples through a 0.45 µm glass fiber filter (Pall Life Sciences, Ann Arbor, MI) and following the procedure recommended by EPA method 160.2.

Statistical analysis of data was performed using *R project software*. A *t-test was conducted to analyze the difference between the means of the* enterococci total concentration and the tylosin-resistant enterococci concentration. The null hypotheses was that there is no difference in the enterococci total concentration and the tylosin-resistant enterococci concentration and statistical significance was determined when $p \leq 0.05$.

Table 1. Enterococci confirmation procedure

	Manure samples	Soil sample	Water samples
Step 1	- Select 6 well-isolated typical and 6 well-isolated atypical colonies from the centers filter paper	- Select 6 well-isolated typical and 6 well-isolated atypical colonies from the centers filter paper	Select 15 well-isolated typical and 15 well-isolated atypical colonies from the centers filter paper
Step2	Transfer into a BHI Broth tube and onto a BHI Agar slant (use a sterile inoculating loop or needle)		
Step 3	Incubate broth for 24 ± 2 hours and agar slants for 48 ± 3 hours at 35°C ± 0.5°C.		
Step 4	Transfer a loopful of growth from each BHI Broth tube to BEA, BHI Broth, and BHI Broth with 6.5% NaCl		
Step 5	Incubate BEA and BHI Broth with 6.5% NaCl at 35°C ± 0.5°C for 48 ± 3 hours Incubate BHI Broth at 45°C ± 0.5°C for 48 ± 3 hours (turbidity)		
Step 6	Perform a Gram stain using growth from each BHI Agar slant.		
Step 7	Observe all verification media for growth. Gram-positive cocci grow on BEA (use hydrolyze esculin on BEA and produce a black or brown precipitate) and grow in BHIB with 6.5% NaCl at 35°C ± 0.5°C and BHIB at 45°C ± 0.5°C are verified as enterococci.		

Results and discussion

Results of these experiments demonstrate the occurrence of enterococci and tylosin-resistant enterococci in the environment from agricultural operations administering tylosin at sub-therapeutic levels. Tylosin-resistant enterococci was detected in swine manure, soil samples collected after land application of swine waste, and from water samples collected at the outlet of a tile drain. This confirms the transport of enterococci and tylosin-resistant enterococci from swine waste and into tile drainage systems from lands under no-till management.

Occurrence of tylosin-resistant enterococci in manure, soil and drainage water Enterococci concentrations were highest in swine manure, averaging 2.2×10^6 cfu/100ml between the two simulations. After manure application to the no-till plots, enterococci concentrations averaged 8.8×10^4 but before application concentrations were much lower (Figures 3 and 4). The fraction of resistant enterococci over total enterococci was different between the two experiments. In the spring 2009 experiment, we found all of enterococci were resistant to tylosin in manure samples and in soil samples approximately 75% of the enterococci were tylosin-resistant after land application of manure. However, during the fall 2009 experiment, only 61% enterococci in manure sample were resistant to tylosin and 44% of enterococci in the soil after land application were tylosin resistant.

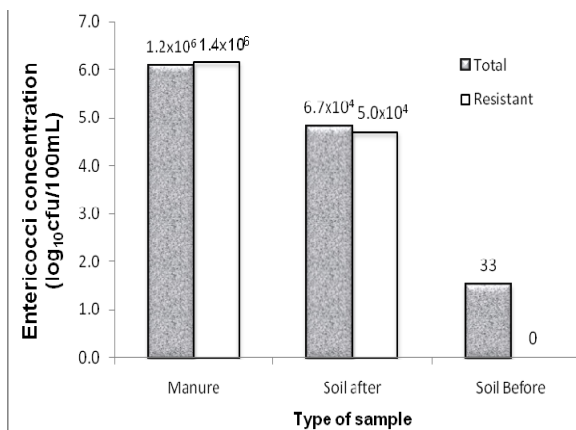


Figure 3. Concentrations of total enterococci and tylosin-resistant enterococci in manure and soil samples in plot 25 during the spring 2009 experiment.

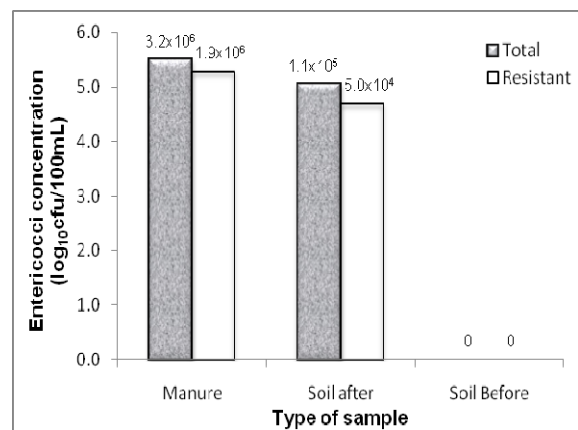


Figure 4. Concentrations of total enterococci and tylosin-resistant enterococci in manure and soil samples in plot 20 during the fall 2009 experiment.

In drainage water, the patterns of total enterococci and tylosin-resistant enterococci were similar in both plots (Figures 5 and 6). During the spring experiment, concentrations peaked at the outlet of the tile drain 80 minutes after the initiation of flow. Peak concentrations for total and tylosin-resistant enterococci were 5.0×10^3 cfu/100 mL and 1.17×10^3 cfu/100 mL, respectively. After the peak, bacteria concentrations decreased sharply until 250 minutes after flow began when total enterococci concentrations become stable and tylosin-resistant enterococci concentrations became nearly nondetectable.

During the fall experiment, two peaks of bacterial concentrations were observed (Figure 6). As reported in the materials and methods section, base flow was present during this experiment prior to the initiation of the experiment. Water sample collection began when the rainfall simulation started. The first peak enterococci concentration occurred 45 minutes after the start of sampling and the second peak occurred 110 minutes after the start of sampling with the second peak being approximately half the concentration of the first peak. In general, enterococci concentrations ranged from 3.3×10^1 to 5.0×10^3 cfu/100 ml for total enterococci and 6.7×10^1 to 1.17×10^3 cfu/100 ml for resistant enterococci. These ranges are almost double the range of enterococci concentrations in drainflow during the spring experiment (1.3×10^1 to 2.57×10^3 cfu/100 ml for total enterococci and 1.3×10^1 to 1.43×10^3 cfu/100 ml for resistant enterococci). This is explained by higher flows, mostly due to the base flow during the fall experiment and perhaps other field conditions that differ between the two plots such as soil type, slope and antecedent soil moisture content. *A simple t-test was used to compare the means of the enterococci total concentration and the tylosin-resistant enterococci concentration. Statistically significant differences were determined for both the spring and fall experiments with $p \leq 0.05$.*

Lower enterococci concentrations in water samples when compared to manure samples are attributed to dilution from the rainfall, bacterial decay and attached to soil particle during the macropore flow migration process, and filtering and retention of microorganisms by the soil during matrix flow. The fraction of tylosin-resistant enterococci were highest in manure samples and lowest in drainage water samples, suggesting that enterococci lose resistance as selective pressure from antimicrobial residues decrease.

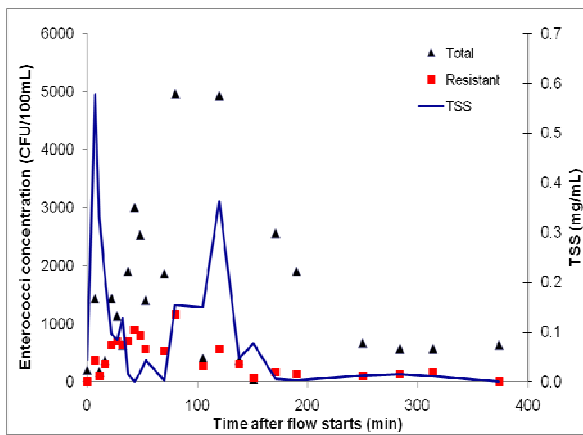


Figure 5. Concentrations of total enterococci, tylosin-resistant enterococci, and TSS in drainage water in plot 25 during the Spring 2009 experiment.

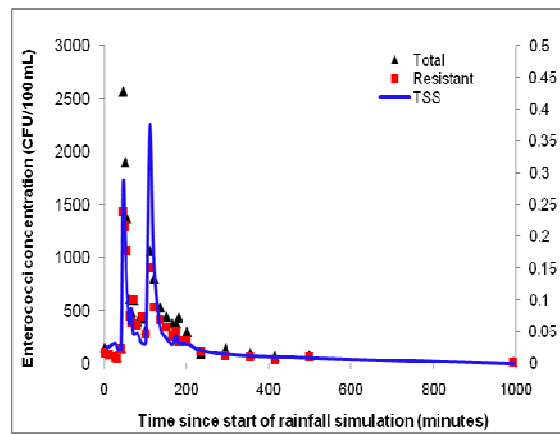


Figure 6. Concentrations of total enterococci, tylosin-resistant enterococci, and TSS in drainage water in plot 20 during the Fall 2009 experiment.

Relationship between enterococci and TSS Due to high base flow during the fall experiment, the range of TSS concentrations is much lower than during the spring experiment. The TSS concentration in plot 20 ranged from 0 to 0.37 g/L, whereas in plot 25, the TSS concentration varied from 0 to 0.58 g/L. The TSS curve matched the time series bacteria concentrations in drainflow (Figures 5 and 6). During the spring experiment, TSS concentrations peaked at the 2nd sampling time because sediment deposited in tile line. Enterococci concentrations, however, continued to increase and peaked 80 minutes after the start of the flow. After enterococci concentrations peaked, the suspended solids in the water samples gradually decreased. The second TSS peak corresponded with the total enterococci concentration peak but lagged the peak tylosin-resistant enterococci concentration. During the fall experiment, the temporal TSS concentrations are similar to the temporal enterococci concentrations. Base-flow prevented the initial TSS peak observed during the spring experiment and the two TSS peaks occurring at 45 and 110 minutes after the start of sampling corresponded to the two enterococci peaks. For enterococci, the first peak is greater than the second peak while the first TSS peak is lower than the second peak. These data suggest a relationship between TSS and enterococci concentrations in drainflow and further statistical analysis is planned to test for correlations between the enterococci and TSS concentrations.

Conclusions

Tylosin-resistant enterococci was detected in manure, soil, and tile drainage water samples collected from two no-till field plots receiving land applied swine waste. Tylosin-resistant enterococci moves from swine waste through macropores and into tile lines. TSS concentrations appear to be closely related to enterococci transport, possibly due to the attachment of enterococci to soil particles during transport. Future analysis will examine relationships between bacteria and TSS concentrations and also investigate hydrologic relationships using flow data.

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