Occurrence and Movement of Antibiotic Resistant Bacteria, in Tile-Drained Agricultural Fields Receiving Swine Manure

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Abstract
The use of tylosin at subtherapeutic levels by the swine industry provides selective pressure for the development of antibiotic resistance in gastrointestinal bacteria. The land application of swine manure to drained agricultural fields might accelerate the transport of pathogen indicators such as enterococci as well as antibiotic-resistant bacteria. The objective of this study was to develop an understanding of the occurrence and transport of antibiotic-resistant enterococci in tile-drained chisel plow and no-till agricultural fields that have received multi-year application of liquid swine manure through injection. Enterococci resistance to tylosin in manure, soil and water samples was investigated phenotypically and compared with samples from control plots treated with urea and ammonium nitrate (UAN). The analysis found that 70% of the enterococci in manure samples were resistant to tylosin. Concentrations of enterococci in tile water were low, and only exceeded the geometric mean for recreational waters 9 times, with 33% of these exceedences occurring in tile flow from the control plots. The results of this study indicate that the occurrence of tylosin-resistant enterococci in tile water is low, found in only 16% of samples, in both control plots and plots receiving fall manure application.

Keywords
Swine manure, tylosin, tylosin-resistant enterococci, tile drainage

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Abstract. The use of tylosin at subtherapeutic levels by the swine industry provides selective pressure for the development of antibiotic resistance in gastrointestinal bacteria. The land application of swine manure to drained agricultural fields might accelerate the transport of pathogen indicators such as enterococci as well as antibiotic-resistant bacteria. The objective of this study was to develop an understanding of the occurrence and transport of antibiotic-resistant enterococci in tile-drained chisel plow and no-till agricultural fields that have received multi-year application of liquid swine manure through injection. Enterococci resistance to tylosin in manure, soil and water samples was investigated phenotypically and compared with samples from control plots treated with urea and ammonium nitrate (UAN). The analysis found that 70% of the enterococci in manure samples were resistant to tylosin. Concentrations of enterococci in tile water were low, and only exceeded the geometric mean for recreational waters 9 times, with 33% of these exceedences occurring in tile flow from the control plots. The results of this study indicate that the occurrence of tylosin-resistant enterococci in tile water is low, found in only 16% of samples, in both control plots and plots receiving fall manure application.

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Introduction

A variety of antimicrobials are used in the swine industry at therapeutic levels for disease eradication and at sub-therapeutic levels as prophylactic and growth promoting agents added to swine feed. These pharmaceuticals can boost the daily growth of pigs and reduce animal death rates at swine facilities, thereby enhancing overall production efficiency and increasing profitability. However, antibiotics and metabolites are also frequently detected in urine and feces from pigs (Mackie et al. 2006). Reports of antibiotics and antibiotic-resistant bacteria in surface water and groundwater around commercial swine operations have focused regulatory and public attention on the swine industry. This is part of a broader concern about the release of human and veterinary antibiotics, their metabolites, or drug-resistant enteric bacteria into the environment and the potential development of a reservoir of organisms that can transfer resistance to pathogenic bacteria (Dantas et al. 2008).

Selection pressure from administration of antibiotics results in the emergence of resistant bacteria in the enteric flora of the animal. Although resistant bacteria can occur in waste from swine not receiving antibiotics, those that are administered antibiotics can discharge 30 to 70% more resistant organisms (Langlois et al. 1986; Jackson et al. 2004). The age of pigs, housing system, and moving stress can also impact the discharge of resistant organisms (Cromwell 2002). Land application of swine manure is a significant route by which antibiotic resistant bacteria can enter into the environment (Kümmerer 2009). In drained landscapes, these agents can seep below agricultural fields and move into subsurface tile drains that connect to surface ditches or streams. Bacteria follow preferential flow paths with reduced residence time in no-till fields versus chisel-plow plots via transport through macropores (Cullum 2009) and via surface runoff (Soupir et al. 2006).

Contamination of surface water and groundwater by antibiotic resistant organisms from swine waste is of significant environmental concern because i) antimicrobial resistance can be more frequent in pathogenic bacteria than in indicator organisms such as *E. coli* (Boerlin et al. 2005); ii) antibiotic resistant bacteria can also harbor virulence genes (Boerlin et al. 2005); and iii) resistant bacteria can remain viable in the environment for several weeks to months (Marshall et al. 1990). Recent studies have reported the presence of bacteria that are resistant to multiple antibiotics above expected background concentrations in surface water and groundwater samples collected near swine farms (Campagnolo et al. 2002; Sayah et al. 2005; Anderson and Sobsey 2006). Elevated levels of resistant bacteria and a broad range of resistance genes have been identified in lagoon water and groundwater underlying swine production facilities (Chee-Sanford et al. 2001; Koike et al. 2007; Mackie et al. 2006). Environmental samples probed for the highly pathogenic *E. coli* O157 revealed high frequencies of resistance to tetracycline, sulfamethoxazole, and cephalothin in isolates obtained from swine operations (Schroeder et al. 2002).

The occurrence of antibiotic resistant bacteria near swine operations is widely reported. However, very little is known about their release from tile-drained fields receiving swine-manure application. Tile-drainage has the potential to significantly accelerate the transport of these organisms to surface water. Other factors such as soil properties (Onan and LaPara 2003), waste characteristics (Mackie et al. 2006), and manure management practices (Chenier and Juteau 2009) can also influence transport and die-off of bacteria from treated soils. These processes can control the movement of resistant bacteria and resistance genes to subsurface tile drainage waters. Data from full-scale field experiments can, therefore, provide timely and valuable information about the relative retention and transport of antibiotic resistant bacteria in manure-amended, tile-drained agricultural fields in the corn/soybean cropping systems prevalent in the Upper Midwest. The goal of this research project was to investigate the...
occurrence and transport of antibiotic-resistant enterococci in tile-drained agricultural fields that have received multi-year application of liquid swine manure through injection.

Materials and Methods

Study Site and Sample Collection

Four plots were identified for sampling at Iowa State University’s Northeast Research Farm near Nashua, IA. These agricultural plots are instrumented with a subsurface water quality monitoring system operational since 1988. Each one-acre plot is drained separately with subsurface drain lines installed in the center of the plot at a depth of 1.2 m below ground surface and a drain spacing of 28.5 m. Cross flow between plots is avoided by border drains (Kanwar 1999). Central drainage lines from each plot are connected to individual sumps equipped with a 110-volt effluent pump, water flow meter, and an orifice tube to collect water samples (Kanwar 1999). Flow meters record subsurface drainage flow continuously as a function of time and are recorded weekly while the tile lines are flowing.

The four plots were selected based on manure application rate, tillage practice, and crop rotation. The selected plots encompass two tillage practices, chisel plow and no-till, and manure was applied to one of each tillage type while the second of each type received UAN and served as a control for assessing background levels. Manure was applied to the plots on October 28, 2010. The manure was from a commercial finishing facility currently feeding tylosin at sub-therapeutic levels of 40 gram/ton for growth promotion. The manure was applied to field plots at agronomic rates equivalent to 150 lb N/acre via injected with a shank forming a band of treated soil.

Soil samples were collected on October 29, 2010. Six composite soil samples were collected from each manure plot, three from the manure band and three from area between the manure bands. Each sample consisted of a composite of 3 cores to 15 cm depth. Three composite samples were also collected from the control plots. Samples were collected in gallon plastic bags and placed on ice in a cooler and transported back to the Iowa State University Water Quality Research Lab. Samples were mixed using surface sterilized spatulas. Subsamples were removed for analysis of total enterococci, tylosin-resistant enterococci, and moisture content. The remaining sample was frozen. A second and set of soil samples were collected on April 13, 2011 and processed using the same sample collection and analysis protocol as in the initial sampling. The manure bands were flagged in the fall to allow accurate repeat sampling.

Enterococci and Enterococci Resistance to Tylosin

Manure, soil, and water samples were assayed for enterococci and enterococci resistant to tylosin by the membrane filtration method (APHA and WEF 1998). Enterococci were selected as target organisms because of their role as indicators of pathogens, their use in regulatory standards, and their use in past studies on bacteria transport. Most importantly, enterococci are gram positive organisms which are targeted by macrolides like tylosin. Furthermore, enterococci were found in nearly 80% of surface water samples in central Iowa (Ilsen et al. 2011) promulgating the use of enterococci for drainage water analysis. Soil and manure samples were diluted with phosphate-buffered water prior to filtration. Concentrations of total and tylosin-resistant enterococci were determined by enumerating colony forming units (cfu) present on mEnterococcus (mE) agar (Difco, Detroit, MI) without antibiotics (control) and mE agar infused with tylosin tartrate at 35 mg/L (Kaukas et al. 1988; CLSI 2010; FDA 2009). Agar was infused with 1 mL of a stock solution made from tylosin tartrate (Sigma-Aldrich, St. Louis, MO) by dissolving 410 mg in 10 mL of 50% methanol as recommended in previous studies.
(Benning and Mathers 1999; Kaukas et al. 1988). The 410 mg accounts for 85% purity of tylosin tartrate. After filtration, the filters were placed onto the each of the media types and incubated at 35 ± 0.5°C for 48 hours. All samples were analyzed in triplicate. Colonies enumerated on control media accounted for the total enterococci population and colonies enumerated on tylosin infused media accounted for tylosin-resistant enterococci.

Results and Discussion

The data from this study showed the occurrence and movement of antibiotic resistant bacteria in tile-drained agricultural fields following the application of swine manure. The following sections discuss the detected levels of enterococci and tylosin-resistant enterococci. The concentrations were the lowest in tile-drainage water and the highest in the swine manure.

Occurrence of tylosin-resistant enterococci

The average concentrations of total and tylosin-resistant enterococci in manure samples were 56,571 (±30,406) cfu/gdw and 39,653 (±39,653) cfu/gdw, respectively. Seventy percent of the enterococci in manure samples were resistant to tylosin. The concentrations of enterococci and tylosin-resistant enterococci are listed in Table 1. On average, 5% of the bacteria from the fall soil samples and 33% of spring samples collected within the manure bands were resistant to tylosin. These results compare to a study by Onan and LaPara (2003) where 69% of the bacteria from manure samples were resistant to tylosin and between 5.8-6.7% of the soil bacteria on plots where manure had been applied were resistant. A decrease tylosin over time has been reported (Sassman et al. 2007; Clay et al. 2005); winter, therefore, provides adequate time for this decrease to occur. Due to bacterial die-off, as modeled by Benham (2006), enterococci concentrations and also resistant enterococci concentrations were therefore expected to decrease in the soil over the winter. In general, concentrations in the no-till plots decreased over the winter, but concentrations in the chisel plow plots did not.

Table 1: Soil Bacterial Concentrations for Total and Tylosin-Resistant Enterococci

<table>
<thead>
<tr>
<th>Tillage</th>
<th>Manure Location</th>
<th>Total Enterococci (cfu/gdw)</th>
<th>Tylosin-Resistant Enterococci (cfu/gdw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fall Samples</td>
<td>Chisel Plow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>11 (±11)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manure No-Band</td>
<td>37 (±35)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manure Band</td>
<td>85 (±8)</td>
<td>0</td>
</tr>
<tr>
<td>No-till</td>
<td>Control</td>
<td>71 (±14)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manure No-Band</td>
<td>830 (±7)</td>
<td>33 (±31)</td>
</tr>
<tr>
<td></td>
<td>Manure Band</td>
<td>822 (±67)</td>
<td>57 (±15)</td>
</tr>
<tr>
<td>Spring Samples</td>
<td>Chisel Plow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>57 (±54)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manure No-Band</td>
<td>41 (±16)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manure Band</td>
<td>316 (±360)</td>
<td>69 (±93)</td>
</tr>
<tr>
<td>No-till</td>
<td>Control</td>
<td>12 (±4)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manure No-Band</td>
<td>31 (±10)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Manure Band</td>
<td>175 (±120)</td>
<td>76 (±90)</td>
</tr>
</tbody>
</table>

Tile drainage water showed a wide array of enterococci concentrations relative to time (Figure 1). Bacteria concentrations were expected to decrease over time after manure application. This was not the case as a decrease was observed in both total and tylosin-
resistant enterococci the first few weeks of sample collection, total enterococci concentrations peaked in late June in three of the four plots.

The US EPA standard for enterococci in recreational water is 33 cfu/100 ml. In the water samples collected in this study, nine samples exceeded this water quality standard. Four of the nine exceedences occurred early in the tile-flow season, most likely due to the first “flushing” of the system since the previous fall. Of the exceedences, 33% were detected in tile flow from the control plots. Enterococci in tile lines draining control plots may indicate that background wildlife sources are contributing to the total enterococci load as found by Marshall (1990). The higher values later in the season occurred following rainfall events, suggesting that these were a function of the increased flow after a period of lower flow. Because the samples were collected as grab samples, they represented an instantaneous point in time and may not be representative of an average concentration at the tile line discharge. Further, samples collected soon after precipitation events are likely from the falling limb of the hydrograph which may differ from the mean concentration during the storm event. The total tylosin resistant enterococci concentrations in the tile water were low and were rarely detected. No resistant enterococci were found in the chisel plow control plot, and only one colony was detected in the chisel plow plot with manure applied.

![Figure 1: Total enterococci concentrations in tile water in cfu/100ml. The EPA Water Quality Standard for recreational water samples (<33 cfu/100 mL for a 30 day geometric mean) is shown for reference.](image)

Of the total number of enterococci colonies enumerated from tile water (1359), only 4 colonies were detected that were resistant to tylosin. In total, there were 44 samples that were positive for enterococci, and 16% of those samples contained bacteria resistant to tylosin. These results compare to previous studies on the occurrence of antibiotic resistant bacteria in the manure, soil and tile water. Resistant bacteria occurred naturally in organic manure (Jindal et al. 2006) and in soil (Onan and LaPara 2003). In the same study, Onan and LaPara (2003) found that an increased proportion of tylosin-resistant bacteria were detected in fields amended with cattle, swine and chicken manure (25% resistant) associated with sub therapeutic use of antibiotics compared with fields where only organic manure was applied (2% resistant). Of the water samples from the control plots in this study, 9% of the samples had detectable resistant enterococci. Because tylosin is a natural fermentation product of *Streptomyces fradiae*, it is possible that tylosin is present naturally in the soil providing an explanation for the tylosin and
tylosin-resistant bacteria detects in the soil and water samples collected from the control plots. The use of tylosin at subtherapeutic concentrations will select and increase resistance to macrolides in enterococci living in the intestinal tract of pigs (Aarestrup and Carstensen 1998), but the results of this study indicate that a very small fraction of these resistant enterococci are transported to tile waters.

It was expected to find more enterococci in tile waters from the no-till plots due to advanced macropore development. No-till fields typically have increased macropore connectivity due to more worm holes and root channels than a tilled fields; this enhances movement of water and enterococci through soil (Shipitalo et al. 2000). However, the results were mixed, as of the 9 data points over the EPA limit, three come from the no-till control, three come from the chisel plow with manure treatment, two come from the no-till with manure, and one from the chisel control. This is likely attributed to the drier conditions that the research farm experienced. 2011 saw less rainfall than average for the area, and therefore tile flow was less than the 5-year average of total flow, as shown in Table 2. This indicates that quantity of bacteria in tile drainage water might be less than expected during a year with higher flow. Therefore, the data potentially underestimates the concentration of tylosin-resistant enterococci in tile water during an average flow year, where it is likely that there would have been more transport through the tile lines and/or macropore flow in the no-till plots over chisel plow plots.

Table 2: Tile Flow Data

<table>
<thead>
<tr>
<th>Plot</th>
<th>Total Tile Flow (m³)</th>
<th>Percent Difference From Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2011</td>
<td>5-year average (2007-2011)</td>
</tr>
<tr>
<td>Chisel Plow with Manure</td>
<td>147.8</td>
<td>371.8</td>
</tr>
<tr>
<td>Chisel Plow with UAN</td>
<td>158.2</td>
<td>289.5</td>
</tr>
<tr>
<td>No-Till with Manure</td>
<td>202.4</td>
<td>305.1</td>
</tr>
<tr>
<td>No-Till with UAN</td>
<td>105.4</td>
<td>394.8</td>
</tr>
</tbody>
</table>

There are other factors that might impact the leaching of antibiotic resistant bacteria from waste-amended soils including waste characteristics or manure storage. The manure was stored in a waste lagoon for up to nine months before it was applied, leaving a potentially large window to facilitate conditions in which the bacteria and tylosin would dissipate in the system, similar to previous findings (Kolz et al. 2005). Based on the findings of Teeter and Meyerhoff (2003) as described previously, the time the manure spent in the lagoon presumably has a direct relationship to the final concentration of bacteria and tylosin-resistant bacteria applied on the field.

**Conclusion**

This study illustrates the occurrence of total and tylosin-resistant enterococci in swine manure, soil and tile water from drained agricultural fields. In the three matrices, both the total and tylosin-resistant enterococci were highest in the swine manure, and lowest in tile drainage water. Tylosin-resistant bacteria were detected in elevated concentrations in soil in the manure injection zone, compared to soil outside the manure band or in soil not treated with manure. Concentrations of enterococci in tile water are very low, and only exceeded the geometric mean for recreational waters 9 times, with 33% of these exceedences occurring in tile flow from the control plots. The results of this study indicate that the occurrence of total and tylosin-resistant enterococci in tile water is low in both control plots and plots receiving fall manure application.
Acknowledgements

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