A Unique Approach to Managing the Problem of Antibiotic Resistance

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A Quick Review

- The sources of antibiotics
  - Release of antibiotics from hospitals and pharmaceutical companies into wastewater
  - Run-off of antibiotics from feedlots and fields where feedlot manure was applied
- The potential problems associated with antibiotics in our waterways
  - Possible toxic effects of antibiotics
  - Selection for antibiotic resistant organisms
- Possible solutions
  - Phytoremediation
Possible Solutions

- Regulate the subtherapeutic use of antibiotics in livestock production
- Place stiffer regulations on the proper disposal of antibiotics from hospitals and pharmaceutical companies
- Put research money into the development of new antibiotics
- Stop or reduce the flow of antibiotics and antibiotic resistant genes into the environment
The Goal

- Eliminate the problem at the source
- Stop spread of antibiotics and antibiotic resistant genetic elements into the environment
- Reduce selective pressure for transfer of antibiotic resistant genetic elements

Can composting feedlot manure help us achieve this goal?
Field Study 2004: The Setup

- 2 management treatments
  - Stockpiling
  - Composting
- 4 piles per treatment
  - 1 control pile – no antibiotics
  - 3 experimental piles – the following antibiotics were spiked into the piles at a concentration of ~300ug/kg manure:
    - Monensin
    - Chlortetracycline
    - Tylosin
Field Study 2004
Sampling method and schedule

- Sampling was done three times per week from the start of the study in late September to late November.
- Another sample was taken in mid-February.
- Samples were obtained by using a modified hay bale corer to take 8-10 cores from the center of each of the piles.
- These cores were combined in a bag for a representative sample.
Sample Analysis

Analysis has focused on the tetracycline class of antibiotics. Samples were analyzed by the following methods:

- **Analytical Chemistry Methods**
  - Quantification of CTC using HPLC/MS/MS

- **Traditional Culturing Methods**
  - Enumeration of CTC-resistant organisms

- **Molecular Methods**
  - Quantification of tetracycline resistant genes using quantitative real time polymerase chain reaction (Q-PCR)
Schematic Diagram of Sample Extraction

Sample (Slurried)

Pre-Extraction

Clean-up (SPE)

Evaporation and Reconstruction

HPLC/MS/MS Analysis

Chlortetracycline: McIlvaine Buffer Solution (pH 4.0)

Nitrogen Gas Water Bath (50°C)
50μl Sample + 70μl mobile phase A
# High Performance Liquid Chromatography

## Tandem Mass Spectrometry (HPLC/MS/MS)

| Equipment | HP 1100 HPLC equipped with Thermostatted Auto Sampler and variable UV detector  
ThermoFinnigan LCQ Duo ion trap mass spectrometer  
Xterra MS C\textsubscript{18} (2.1×50mm, 2.5\textmu m pore size, end-capped) |
|---|---|
| Optimized HPLC Condition | Column Temperature (°C)  
Flow Rate (ml/min)  
Mobile Phase Conditions:  
Mobile Phase A (99.9% DI+ 0.1% Formic Acid)  
Mobile Phase B (99.9% ACN + 0.1% Formic Acid) |
| Chlortetracycline | 15  
0.32  
A: 96% + B: 4%: 0 (min) \(\Rightarrow\) A: 70% + B: 30%: 29 (min) \(\Rightarrow\) A: 96% + B: 4%: 30 (min) |
| Optimized MS Condition | Nitrogen Gas used for drying and nebulizing  
Spray Voltage – 4.5V  
Capillary Voltage – 21V  
Capillary Temperature - 165°C |
Tetracycline Concentration

Initial Rapid Degradation within 10 days

More Rapid Degradation in Composting

Needs to be compared with antibiotics resistance bacteria profiles

Tylosin and Monensin will be evaluated
Percentage of Antibiotic Resistant CFUs in Compost Piles

![Graph showing percentage of antibiotic resistant CFUs in compost piles over time](chart)

- LB-Tyl compost -Ab
- LB-Tyl compost + Ab
- LB-Mon compost -Ab
- LB-Mon compost + Ab
- LB-CTC compost -Ab
- LB-CTC compost + Ab

Average CFU over time (days):

- 0% to 40%
- Time points: 1, 5, 12, 19, 26, 33, 40, 42
Percentage of Antibiotic Resistant CFUs in Stockpiles

Percent of microbes found in stockpiling that were resistant to Ab

- LB-Tyl stockpiling -Ab
- LB-Tyl stockpiling +Ab
- LB-Mon stockpiling -Ab
- LB-Mon stockpiling +Ab
- LB-CTC stockpiling -Ab
- LB-CTC stockpiling +Ab

Time (days) range from 1 to 42.
The Tet Family Tree

- **Efflux**
  - Tet A, B, C, D, E, F, G, H, J, Z, 30

- **Ribosomal Protection**
  - otrA, tet M, O, B/P, Q, S, T, W

- **Enzymatic Alteration**
  - Tet X

* This is not a comprehensive list, there are over 38 known tetracycline resistant genes. This lists the tet genes that have been more commonly studied over the past few years. 9 of the total 38 genes were discovered in the last 4 years.
Quantifying Tetracycline Resistant Genes

- Use a method called quantitative real-time polymerase chain reaction (Q-PCR)
- Works like regular PCR, except that there are fluorescent dyes used to measure the product of the PCR
- Each reaction tube is controlled separately and the fluorescence is measured over time
- This fluorescence can be related to the amount of a gene present by creating a calibration curve for each protocol
Calibration Curve

\[ y = -4.9623x + 61.753 \]

\[ R^2 = 0.9971 \]
Monitoring \([\text{tet}\text{W}]/[\text{16S}]\) over time
Monitoring \([tetO]/[16S]\) over time
What about these two genes causes them to behave so differently?

- \(tet(W)\) and \(tet(O)\) are commonly found in the bacteria of ruminant animals.

- Bacteria possessing the \(tet(W)\) gene could be more able to make the transition from the animal’s gut to the environment than those bacteria containing the \(tet(O)\) gene.

- The rapid transfer of \(tet(W)\) has been documented.

- \(tet(W)\) gene could be transferred more easily and thus at a higher frequency than the \(tet(O)\) gene.
Conclusions from Field Study ‘04

- Antibiotic concentrations are decreasing in both treatments
- Some degradation of CTC is likely microbially mediated
- When the selective pressure for tetracycline resistance has passed, tetracycline resistant genes seem to decrease
- Composting does seem to increase the concentration of $tet(W)$ initially, but then decrease during the curing phase
- Composting could still be used as a treatment method
  - Degradation of tetracycline
  - Removal of pathogenic bacteria
  - Improvement of quality and texture for land applications
  - Economic value as a marketable product
Field Study 2005
From Fall ‘04 to Summer ’05: What Have We Learned Along the Way?

- Making a few large, long windrows that are divided into sampling sections instead of several small piles
- Sampling once a week rather than 3 times a week for consistency and increased productivity
- Turning the compost once a week, based on my sampling schedule to improve consistency and to allow for sampling when compost is most stable
- Using a compost turner to turn and mix the pile instead of a front-end loader
- Slurry samples with sterile water to make samples more homogeneous, then use this slurry for all analysis to follow
- Focusing time and efforts on molecular analysis of the samples rather than traditional culturing methods
Future Work

- Right now efforts are focused on analyzing the levels of the \textit{tetX} gene in the samples from the Fall 2004 study.
- Also suppression studies are being done to determine the matrix effects the DNA extract may have on the amplification of DNA targets.
- The samples from Summer 2005 will be analyzed for several antibiotics and several genes including \textit{tetW}, \textit{tetO} and \textit{tetX}.
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The End

Questions???
Other Information
The Problem

- CDC reports that each year 2 million patients acquire nosocomial infections
- 90,000 die from these infections
- 70% of the infection-causing bacteria are resistant to drugs normally used to treat the infection
- Growing concern that resistant pathogens could be used as biological warfare agents

http://www.cdc.gov/drugresistance/healthcare/problem.htm
The Sources

Commonly Identified Sources:

- Overuse of antibiotics in hospitals
- Subtherapeutic use of antibiotics in livestock feed

Other Sources:

- Release of antibiotics from hospitals into wastewater
- Run-off of antibiotics and antibiotic resistance genes from feedlots and fields where feedlot manure was applied
A Little Bit of History

- 1948: The first tetracyclines (CTC and OTC) were discovered
- 1952: Tetracycline was first used clinically
- 1950’s: Farmers first began adding antibiotics to their feed (medicating their feed) to increase weight gain in their livestock
- 1956: Tetracycline resistance was first detected
Composting vs. Stockpiling: What’s the Difference?
When done properly, the compost will go through three phases:

1. **Mesophilic Phase**: temperatures range from 20-40°C. phase where simple, easy to degrade compounds are metabolized by the microorganisms.

2. **Thermophilic Phase**: thermophilic bacteria take over, intense microbial activity heats the pile above 40°C to a maximum temperature around 60-80°C. This stage is important for killing pathogens and plant seeds.

3. **Curing Phase**: cooler, slow process that must occur to remove compounds that cause bad odors and that may cause problems with plant growth.

Composting vs. Stockpiling: What’s the Difference?