RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Relationship between Antibiotics in Water and Rates of Antimicrobial Resistance Gene Transfer
NPB #04-159

Investigator: Randall S. Singer, DVM, MPVM, PhD

Institution: University of Minnesota

Date Submitted: January 12, 2006

Abstract:

There is growing concern about the fate of antibiotics and antibiotic residues in the environment. These compounds can potentially have selective effects on bacteria, thus amplifying the resistance gene pool. Although measured levels of these antibiotics in waterways associated with swine operations have been low, they may still be high enough to influence the persistence and dissemination of antibiotic resistance. The objective of this study was to correlate the effects of varied concentrations of tetracycline in water samples with the rate at which antibiotic resistance develops in the bacterial population in the water.

We designed a set of experiments for evaluating the biological effects of low levels of antibiotics in water. We established a system in the laboratory that uses chemostats, also known as bioreactors, to study microbial changes over time. Three different bioreactors were used, and each contained river water. One bioreactor served as the control to which no antibiotics were added. The other two bioreactors contained different levels of chlortetracycline (CTC). When followed over time, the total level of bacteria in the three bioreactors was about the same. Following 5 replicates of this experiment, the general trends are consistent: chlortetracycline at low levels does not appear to select for bacterial populations that are resistant to tetracycline. This experiment needs to be repeated for additional antibiotics, and then the findings from these studies need to be validated through a variety of field studies.

Introduction:

The increasing rate of development of bacterial resistance to antimicrobials has been well-documented. This development of resistance has resulted in human and animal bacterial pathogens that are refractory to many forms of treatment currently available. When trying to solve this extremely complex problem, we naturally look for those activities that are major contributors to the loss of antibiotic efficacy. Antibiotic use is the major selection pressure influencing this situation and its effects are manifested in many different populations and settings, including animals, plants, humans, and the environment. All these antibiotic uses will add to the cumulative selective pressures exerted on bacteria.

A growing concern is the presence of these antimicrobial compounds in the environment. The discharge of wastewater from animal agricultural facilities, human sewage treatment plants, hospitals and pharmaceutical plants has been associated with increased levels of zoonotic pathogens as well as increasingly resistant and virulent organisms. Antibiotics are often discharged from these sites, and these antibiotics in the environment

can act as a selection pressure further influencing the acquisition and spread of resistance genes. Although antimicrobial compounds can be found in the environment, studying the effects of these compounds on the dissemination and persistence of antimicrobial resistance is not easy. In part, the way in which we measure antimicrobial resistance will affect our ability to discern the effects of these antimicrobial compounds.

The ultimate goal of applied antimicrobial resistance research is often to quantify the association between specific selection pressures and their influence on antibiotic resistance over varying spatial and temporal scales. For example, does the use of a specific antibiotic in animal agriculture increase the amount of resistance genes in bacterial populations over short and long-term time scales? If so, does this increase have an affect on human and animal health? Many different bacterial populations can be under the same set of selection pressures, and there is the potential for resistance genes to be shared among a diversity of bacteria. This project will improve our understanding of the effects that low-level antibiotics in water have on resistance levels and resistance dynamics in bacterial populations.

Objective:

The overall objective of this proposal is to assess the impact of antibiotics in water on the spread and persistence of antimicrobial resistance genes. To accomplish this, we must utilize various means of quantifying the effects of the antibiotic as a selection pressure. This project had the following main specific aim:

1) Correlate the effects of varied concentrations of tetracycline in a river water sample with the rate at which antibiotic resistance genes are mobilized among bacteria in the sample.

Materials and Methods:

Experimental Design. The complete project will consisted of 5 replicate laboratory experiments. For each experiment, Mississippi River water was used. We added known concentrations of tetracycline into the water samples. In each of the 5 laboratory experiments, the effects of varying concentrations of tetracycline on the rate of resistance gene transfer was evaluated. We initially set up 3 chemostats (bioreactors) and added 500 mL of river water to each. We then mixed the nutrients for LB broth into the bioreactor to make a broth that was at 1/10 normal concentration. Aeration was connected from a medical grade compressed air tank, and the three bioreactors were allowed to grow for 2 days. On the third day, if the optical density of the water in each bioreactor was ~ 0.4-0.5 the nutrient broth feed tanks were started. These tanks were filled with LB broth, again at 1/10 normal concentration, and the flow into the bioreactors by way of a peristaltic pump system was set to 590 ml per day (which is about the maintained volume of the bioreactors). The bioreactors were allowed to grow for one additional day with the pumps on. The following day was considered Day 0, and antibiotic was added on this day. Chlortetracycline (CTC) wasl be added to two of the three bioreactors each day beginning on Day 0 at a dose of 10 ug / L and 1000 ug / L, both environmentally-relevant antibiotic concentrations. Samples were taken from each aliquot on each day of the 10-day trial.

Plate Count Assays: Plate counts of each bioreactor were assayed from the original river water sample and then daily, from Day 0 to 10, using LB agar, LB agar with 20 µg/ml of chlortetracycline, and LB agar with 8 µg/ml of erythromycin. All plate counts were performed in duplicate. The LB plates were grown at 30°C for 24 hrs.

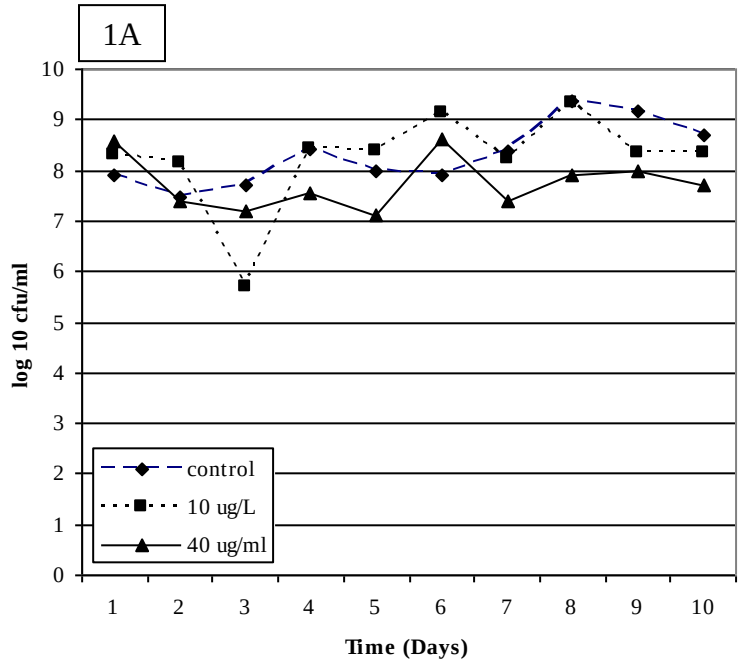
Colony Screen and Probe: A sample of water was taken from each bioreactor on each day and used to inoculate Entercoccosel broth. This was grown at 45°C for 24 hrs. The broth was then plated on m-enterococcus agar plates (with and without antibiotics as described above), and the plates were incubated at 37°C for 24 hrs. Three randomly selected colonies from each bioreactor on each day were evaluated for antimicrobial susceptibility using Sensititre CMV1-AGPF plates in a broth microdilution format. In addition, 48 *Enterococcus* colonies from each plate were picked to a grid and used for colony blot hybridization to determine the proportion of colonies that have acquired specific resistance genes. After growth overnight at 37C, the plates were chilled at 4C for several hours. The grids were transferred to Hybond N membranes (Amersham). Bacteria were lysed and fixed on the membranes. Gene specific probes that have been prepared by PCR amplification were digoxigenen-labeled according to manufacturer's directions using the Genius kit (Boehringer Mannheim). These probes have been designed to detect the specific resistance genes *ermB, tetM* and *tetS*. Hybridization was performed according to the manufacturer's instructions.

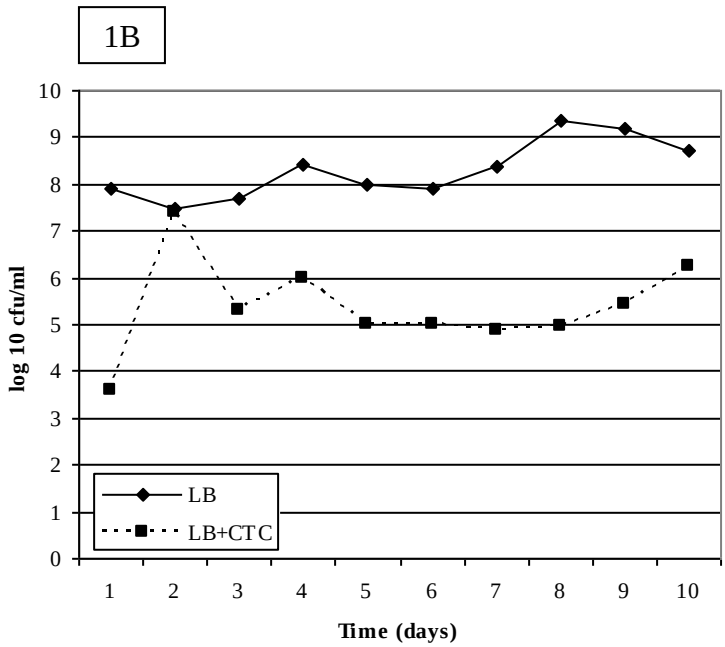
Quantify Antibiotics: The amount of tetracycline present in the samples was measured with a commercially available ELISA kit. The approach followed manufacturer's instructions as well as suggestions made in our previous work. We have shown previously that the detection limit of this assay is > 0.10 µg/L, which is an environmentally significant concentration.
Statistical Analysis: We used analytical models relating the resistance outcome measurements to various explanatory variables. The significance of each variable, model selection, and the overall fit of these models was assessed with standard approaches that we have used extensively.

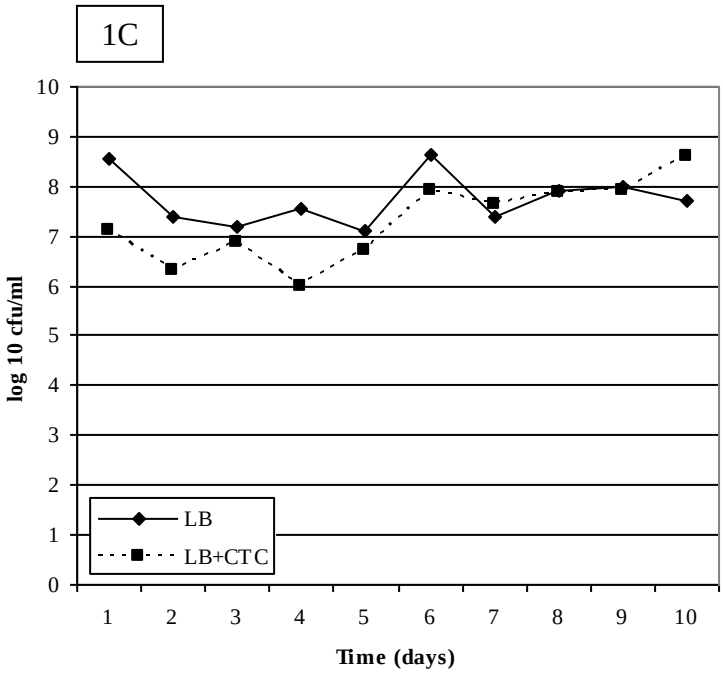
Results:

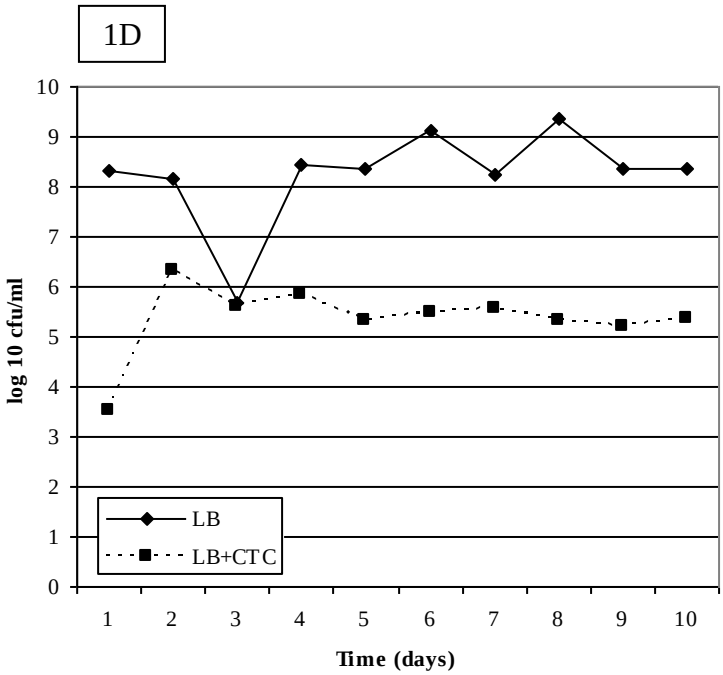
We have established a system in the laboratory that uses chemostats, also known as bioreactors, to study microbial changes over time. Three different bioreactors were used. One bioreactor served as the control to which no antibiotics were added. The microbial population in the river water was followed over time without any additional selection pressure created by antibiotic administration. However, in the other two bioreactors, chlortetracycline (CTC) was added daily. In one of these, 10 ug / L was added twice daily. This is a biologically relevant level that has been observed in the waterways of the U.S. In the other bioreactor, either 40 ug / ml or 1000 ug / L of chlortetracycline was added twice daily. 40 ug / ml is a therapeutic dose of tetracycline. When followed over time, the total level of bacteria in the three bioreactors was about the same (Fig. 1A). The graph shows the total bacterial count when a sample of the water was grown on LB agar. Thus, the antibiotic is not affecting the total bacterial level of the system. Fig. 1B shows the difference in the total bacterial count in the control bioreactor when the water sample is plated on LB agar with and without chlortetracycline (at 20 ug / ml). There is a large reduction in the total bacterial count on the LB+CTC plates indicating that a large percentage of the population is susceptible to chlortetracycline. Conversely, Fig. 1C shows the difference in the total bacterial count in the 40 ug / ml bioreactor when the water sample is plated on LB agar with and without chlortetracycline (at 20 ug / ml). In this case there is no measurable difference between the LB agar with and without CTC indicating a large percentage of the population is now resistant to chlortetracycline. It would thus appear that CTC at 40 ug / ml is capable of selecting for tetracycline resistance. Finally, Fig. 1D shows the difference in the total bacterial count in the 10 ug / L bioreactor when the water sample is plated on LB agar with and without chlortetracycline (at 20 ug / ml). As in the control bioreactor, there is a large reduction in total bacterial count on the LB+CTC plates indicating that a large percentage of the population has remained susceptible to tetracycline. Although there is inter-experiment variability, the general trends are consistent: chlortetracycline at 10 ug / L does not appear to select for bacterial populations that are resistant to tetracycline.

In addition to analyzing the total bacterial counts in the water samples, we also analyzed specific *Enterococcus* isolates that were collected each day of the experiment. Three isolates per bioreactor per day from two of the experiments have been analyzed for antimicrobial susceptibility. In general, the isolates collected from the 40 ug / ml bioreactor have much more resistance to tetracycline (and other antibiotics) than the isolates from the control and 10 ug / L bioreactors, and there is no significant difference between the control and 10 ug / L bioreactors.


1A
log 10 cfu/ml
Time (Days)
control
10 ug/L
40 ug/ml


1B
log 10 cfu/ml
Time (days)
LB
LB+CTC


1C
log 10 cfu/ml
Time (days)
LB
LB+CTC


1D
log 10 cfu/ml
Time (days)
LB
LB+CTC

Discussion:
We tested the ability of chlortetracycline (CTC) at varying levels to select for antibiotic resistant bacteria in river water. We used biologically relevant concentrations of CTC in each experiment. When therapeutic levels of CTC were added to the river water, the proportion of bacteria in the river water increased dramatically and remained significantly higher than the control bioreactor over time. However, when we added low levels of CTC, there was no significant difference between this bioreactor and the control bioreactor. These results were consistent over five replicate experiments. Consequently, it appears that low levels of CTC in river water do not exert a strong selection pressure on bacterial populations. It is possible that combinations of multiple different types of antibiotics in the river water at the same time could exert a cumulative effect on the bacterial population, and thus the results of this study should be considered in light of the fact that only CTC was added to the water. To validate the results of this experiment, additional studies in the field should be conducted to determine how these antibiotics act in the natural environment. In addition, more antibiotics should be tested for their biologically activity at environmentally-relevant levels.

Lay Interpretation:
There is growing concern about the fate of antibiotics and antibiotic residues in the environment. These compounds can potentially have selective effects on bacteria, thus amplifying the resistance gene pool. The swine industry uses considerable amounts of certain antibiotics, such as tylosin and tetracycline. Although measured levels of these antibiotics in waterways associated with swine operations have been low, they may still be high enough to influence the persistence and dissemination of antibiotic resistance. The results of our studies suggested that the low levels of tetracycline observed in the waterways of the U.S. may not have a strong biological effect. In other words, these low tetracycline levels may not be high enough to select for antibiotic resistant bacteria and thus may not pose a threat to human or animal health through an increase in antibiotic resistant bacteria. The results of this study are based on laboratory experiments which may not mimic reality. Consequently, additional studies should be conducted in the field to validate the findings of this study. Ultimately, the results of these studies will help the swine industry manage the potential risks associated with the environmental release of antimicrobial compounds from farms.