

My arrival to this point in my career is the result of a progression that started with my undergraduate work in plant ecology. Two courses in particular, tropical forest ecology and ethnobotany, piqued my interest in how people in tropical cultures interact with the plants in their environment. After graduation I looked for an opportunity to spend an extended period of time immersed in a tropical culture where I could continue to explore this topic. The Peace Corps provided this opportunity along with a chance to make a contribution to the community where I was assigned. Because of my plant background, I was assigned to carry out reforestation and sustainable agricultural projects in a rural community in El Salvador.

During my time there I became aware of the burden placed on communities that are lacking in basic public health infrastructure such as water and sanitation. When basic health needs are not being met it is difficult to focus on less immediate concerns such as deforestation. During Peace Corps training, we were often told that our work should be guided by the needs that are identified by the community. The top priorities in my community were health-related. I did find some success by demonstrating to my community members that reforestation and soil conservation projects can improve community health by protecting the local streams that were their main water supply. However, my experience in El Salvador led to a decision to shift my main focus from ecology to public health.

After completing my Peace Corps service I applied to the Master of Public Health (MPH) program at the University of Minnesota. Focusing on infectious disease epidemiology seemed to be a natural choice given that gastrointestinal, respiratory, and insect-borne diseases were the most salient health issues in El Salvador. During my time in the MPH program I had the opportunity to work at the Minnesota Department of Health (MDH) on surveillance of mosquito and tick-borne diseases in humans. While doing that work, I was able to design and carry out a field study of a mosquito-borne disease, La Crosse encephalitis, which resulted in my Master's thesis. Upon completing the MPH degree, my thesis advisor, Dr. Randall Singer, hired me full time to analyze and publish data from a research study of two viruses that are transmitted by an insect and infect livestock. While working on the publications that resulted from this study, I began to develop the spatial analytical skills that I will use in my PhD dissertation.

Now with my work on antibiotic resistance I have come full circle to my roots in ecology, although I have shifted from plant ecology to microbial ecology. During my time as a PhD student I have also become interested in the issue of validity in health studies. How do we know if we can trust our study results and what can we do to improve the validity of health studies? I have had the opportunity to take two courses that address this topic directly. These three areas of interest (ecology, infectious disease epidemiology, and validity in health studies) are all integrated into the goals of my dissertation proposal.

My goal upon completing my dissertation is to find work that allows me to conduct research in infectious disease epidemiology and to teach. I hope to eventually apply the skills I have learned in graduate school to a research program in Latin America where my interest in infectious disease and public health began.

Quantitative Measurement and Spatial Analysis of Antimicrobial Resistance Gene Load in Agricultural Samples

Background: Antibiotic resistance in disease causing bacteria is widely recognized as a serious threat to public health. Rates of antibiotic resistant infections acquired in hospitals have increased since they were first documented in the 1940's. In recent decades, community acquired resistant infections have increased as well. Some bacteria, such as those that cause certain types of *Staphylococcus* infections, have become resistant to multiple antibiotics, greatly reducing our treatment options for these infections. A significant percentage of antibiotics produced in the United States are used in agriculture for purposes other than treatment of infection, such as growth promotion in livestock. For this reason, the influence that antibiotic use in agriculture may have on the spread of antibiotic resistant infections in humans has received considerable attention. However, it has been difficult to establish whether or not there is a causal link between the two. My research focuses on two of the issues that have obscured attempts to describe the relationship between antibiotic use in agriculture and the risk of resistant infections in humans. The first issue concerns biases in the various laboratory methods that are commonly used to test bacteria for antibiotic resistance and the accuracy of statistical methods used to analyze different types of resistance data. The second issue relates to factors that can affect bacterial resistance to antibiotics at a study location that are not related to antibiotic use at that location. For example, resistant bacteria in agricultural runoff from a farm that uses antibiotics can be carried by water to another farm downstream where antibiotics are not used. Failure to account for factors such as these can lead to biased conclusions about the effect of agricultural antibiotic use on resistance in bacteria.

Most bacteria that live in humans and animals exist in commensal relationships with their hosts; they do not cause disease and in many cases provide benefits to the hosts. Many studies have demonstrated that genetic material, including genes that protect bacteria from antibiotics, can be transferred among widely diverse types of bacteria. Therefore, commensal bacteria may serve as reservoirs of antibiotic resistance if they are able to transfer resistance genes to and from other bacteria including those that cause disease. *How do we estimate the importance of this enormous commensal bacterial population?* Most laboratory methods of testing for antibiotic resistance rely on cultivation methods where bacteria from a sample are grown in nutrient rich media. A subset of bacterial isolates is selected from the culture and is tested for resistance in a variety of ways. A major downside of these approaches is that most bacterial species cannot be grown in the laboratory. *Therefore the majority of types of bacteria in a sample are overlooked when cultivation methods are used to test for antibiotic resistance.* As an alternative to cultivation-based methods, I propose a method of measuring antibiotic resistance that involves extraction of DNA from all of the bacteria in a sample. The quantity of resistance genes in this sample of DNA can be measured using a method known as quantitative polymerase chain reaction (qPCR). With this method, the entire bacterial community of a sample can be studied including those bacteria that cannot be grown in the laboratory.

Many factors can influence antibiotic resistance at a study location that have nothing to do with antibiotic use at that location. Antibiotics are derived from compounds that are naturally produced by many microorganisms in the soil. Genes that provide bacteria with the ability to protect themselves against these compounds have evolved naturally over time, resulting in natural background levels of antibiotic resistance in the environment. As mentioned previously, bacteria are able to share antibiotic resistance genes. Often, multiple resistance genes can be

physically linked, meaning that they move together when genetic material is shared among bacteria. This means that one antibiotic can select for resistance to many different antibiotics, including antibiotics that are not even being used. Additionally, resistant bacteria can be disseminated to new locations by water, wind, or wildlife. Factors such as these can lead us to mistakenly conclude that differences in antibiotic resistance among farms are due to differences in antibiotic use when in actuality these differences might have nothing to do with antibiotic use. It is often the case that these factors affect some locations more than others. In addition, locations that are closer together tend to be more similar to each other, a statistical problem known as spatial autocorrelation that is often overlooked in antibiotic resistance studies. When not accounted for in an analysis, autocorrelation can lead to incorrect statistical conclusions. Techniques exist for accounting for these factors but they have never been applied to ecological studies of antibiotic resistance.

Goals and objectives: My objective is to improve our understanding of how agricultural use of antibiotics affects levels of antibiotic resistance in livestock and agricultural soil. To accomplish this, my project has three Specific Aims: (1) to apply a quantitative PCR assay to an agricultural sample set to determine if this novel approach is better than the standard approach, (2) to compare the accuracy of statistical techniques used to analyze resistance data derived from different laboratory methods, and (3) to combine quantitative PCR data with spatial analytical methods to make more accurate estimates of the relationship between antibiotic levels and antibiotic resistance in agricultural settings.

Design and methodology: For Specific Aim 1, I will test the hypothesis that qPCR provides a sensitive measure of antibiotic resistance changes over time. I will accomplish this aim by applying qPCR methods to a sample set that was collected during a study of antibiotic resistance in dairy cattle from four herds that varied in size, production methods, and antibiotic uses. Fecal samples were collected from the animals every three months over 2.5 years. Information about antibiotic use and production methods was collected at each visit for every animal on the farms. Antibiotic resistance in specific types of bacteria in these samples has already been analyzed using cultivation methods. For the present study, bacterial DNA will be extracted from each sample and the extracted DNA will be used to quantify six genes that protect bacteria from a variety of antibiotics commonly used in animal agriculture. The association between quantitative resistance gene levels and antibiotic use in the animals will be analyzed using standard statistical methods. Data produced by qPCR will potentially allow me to show that animals that have received antibiotics have higher levels of relevant resistance genes in their feces. This relationship was not observed using culture methods on this sample set.

Specific Aim 2 will test the hypothesis that statistical models based on different types of resistance data will lead to different conclusions regarding antibiotic use and resistance. This will be accomplished in two steps. First, I will create a computer simulation of bacterial populations using known (observed) frequencies of resistance genes and of the effect that antibiotics have on this resistance. A sample from each simulated bacterial population will be selected and different data types representing cultivation and qPCR methods will be generated from each sample. These data types will be analyzed in appropriate statistical models. The second step will be to assess the accuracy of the different model types in their ability to estimate the true relationships inherent in the simulated bacterial population. This approach will enable

me to address questions such as: "If the effect of antibiotic use on the gene pool is increased two-fold, how will this be reflected in statistical models using different types of data?"

For Specific Aim 3, I will test the hypothesis that levels of antibiotic resistance genes in agricultural soil are unevenly distributed in space and are associated with antibiotic concentrations in the soil. To accomplish this, soil samples will be collected from an apple orchard where the antibiotic streptomycin has been sprayed for several years to control fire blight, a major bacterial disease of apple trees. Soil samples will be collected from inside the orchard and from adjacent areas not treated with streptomycin. DNA will be extracted from the soil samples and levels of various antibiotic resistance genes, including streptomycin, will be measured by qPCR. Soil samples will also be analyzed for streptomycin concentration and soil characteristics known to influence bacterial activity. A statistical model will be developed to estimate the association between resistance gene levels and antibiotic concentration in the soil while adjusting for other soil characteristics and sample location. This model will be used to predict resistance gene quantities at unmeasured locations on the farm.

Potential significance: In 2001, a task force co-chaired by the CDC, FDA, and NIH identified research priorities in the area of antibiotic resistance. Among these was the need to study microbial ecology and the role of non-disease causing bacteria as reservoirs of resistance.^[1] In 2002, a report from the American Academy of Microbiology echoed this need and emphasized that methods to quantitatively measure antibiotic resistance are needed.^[2] The Specific Aims in this proposal directly address these priorities. The methods I propose in Specific Aim 1 provide a way to quantitatively measure resistance not only in disease causing bacteria but in the entire bacterial population of a sample. The relative accuracies of statistical methods used to analyze different forms of antibiotic resistance data have not been assessed. Specific Aim 2 will help to determine which analytical methods are best suited to answer different questions regarding antibiotic use and resistance. Finally, the importance of accounting for sample location is well-recognized in other fields such as ecology and soil science. Despite growing evidence that bacterial diversity and activity is unevenly distributed in space, this issue is generally overlooked in environmental studies of antibiotic resistance. Specific Aim 3 will provide new information on the spatial distribution of antibiotic resistance genes in the environment.

Progress to date & schedule for completion: I have extracted DNA and performed qPCR assays for 6 genes on over 400 fecal samples. The remaining laboratory work for the first two Specific Aims will be completed in the spring of 2009. I have completed a draft of a manuscript that describes the experiments used to validate the DNA extraction method and qPCR assays. Sample collection for Specific Aim 3 will take place in the spring and early summer of 2009. The laboratory work on these samples will be completed during the summer and early fall of 2009. Development of the simulation and spatial models and preparation of the remaining manuscripts is underway and will be completed by the end of the 2009-2010 academic year. I plan to graduate in the summer of 2010.

Key References:

[1] Interagency task force on antimicrobial resistance, 2001. Retrieved March 11, 2009 from: <http://www.cdc.gov/drugresistance/actionplan/aractionplan.pdf>

[2] Isaacson, R.E. & Torrence, M.E., 2002. Retrieved, March 11, 2009 from: <http://academy.asm.org/images/stories/documents/roleofantibioticsinagricultureenglish.pdf>