UNIVERSITY OF CALIFORNIA

Los Angeles

Antibiotic Resistance in Bacteria Isolated from Commercial Meat Samples

and Air Samples Near Agricultural Sites

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Civil Engineering

by

Helen Mariette Sanchez

2015

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Civil Engineering

University of California, Los Angeles, 2015

Professor Jennifer Ayla Jay, Chair

The rising level of antibiotic resistance is a serious public health issue, posing a global threat to human health. The common practice of applying sub-therapeutic dosages of antibiotics to livestock has been shown to foster the development of antibiotic resistant bacteria (ARB). ARB originating in livestock can reach the general population via multiple pathways: air downwind of animal feeding operations and transportation vehicles, indoor air, soil following land application, surface and groundwater, and retail meat and poultry. Likewise, antibiotic resistance genes (ARGs) confer antibiotic resistance through various mechanisms, and are themselves considered to be emerging contaminants. In Chapter 2, we demonstrate the presence of both ARB and ARGs emanating from cattle production in air. This study is unique in that it is the first to compare resistance in airborne bacteria near conventional and organic beef farms. We used two methods to assess antibiotic resistance: our newly developed high throughput method (HT) for liquid cultures (n= 1295), and a common method, disk diffusion (DD), which involves culturing on solid media. By the HT method, conventional beef production sites showed a greater

average fraction of ARB than organic production for most of the six antibiotics at the low and high concentrations, some with statistical significance. Regular surveillance of ARB and ARGs from beef cattle farms is suggested to detect the spread of ARB and ARG to the community via air trajectories.

This body of work also demonstrates ARB originating from retail meat. In Chapter 3, we present results that suggest that the presence of antibiotic resistant *E. coli* differs depending on the type of poultry production system. In this study, we cultured *Escherichia coli* from retail poultry falling into three categories of farming practices: *Conventional, No Antibiotics,* and *Pristine Organic*. We examined the antibiotic resistance of the *E. coli* isolates (n = 424) by exposing them to seven common antibiotics via a high-throughput, liquid culture-based method. Our findings were that the fraction resistant of the *E. coli* bacteria from the *Pristine Organic* was significantly lower than the *Conventional* and *No Antibiotic* categories, while the latter categories had similar fractions of isolates resistant. It is the first study to suggest that a particular type of organic meat production shows a significant improvement in antibiotic resistance over typical organic brands.

In Chapter 4, the study addresses the gap in literature where there are few studies that address the release of ARGs specifically from poultry CAFOs. To investigate this, upwind and downwind of the farms, bioaerosols were collected and examined for antibiotic resistant genes. To our knowledge, this is the first study to examine air samples collected upwind and downwind of CAFOs for elevated levels of ARGs. Our findings show that there are higher frequency of $bla_{\text{SHV}}$ downwind than upwind in two poultry CAFO sites and also, a higher frequency of $erm$(F) downwind than upwind in three poultry CAFO sites. The higher frequency of ARG detection near downwind versus upwind of poultry farms collectively show the occurrence of antibiotic

resistance in the environment, which may imply potential exposure to ARGs via an air pathway downwind of poultry farm. Regular monitoring and surveillance of ARG from poultry sources is suggested to detect development of airborne antimicrobial resistance that can be spread to surrounding community, especially humans living near farms and workers, via wind air trajectories. The microbiome of family workers can be impacted by these ARG's and research into the public health should be investigated.

The dissertation of Helen Mariette Sanchez is approved.

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**Dedication Page**

I dedicate this dissertation to foremost my mother, Maria Eline Perez, my father, Juan Sanchez

Chavez, and my brother John Joseph Sanchez. They were my A+ team all along and because of

them I was able to finish what I started. They fed me when I was down, they encouraged me to

keep on going when I couldn't , and they loved me. They were my #1 fans cheering me on from

the sidelines coaching me constantly through my ups and downs. I am so grateful and will do

anything for them.  I graduate along with them. I love you all very much. Los quiero mucho.

I would also like to dedicate this dissertation to Edgar Bautista. He was the reason why I finished

because of his constant encouragement and love from the other side. He showed me my mission

in life, which is to become a professor in minority serving institution and help other students

reach their potential and achieve success. I promised him that the last day I saw him and I will do

it. I will always love you too.

Also, I dedicate this to Lucy, my pet hen. I love Lucy.

# Table of Contents

**List of Tables**

**List of Figures**

# I. Acknowledgements

I would like to thank Dr. Jay for always pushing me to me by best and showing me how to be a strong woman in the field of engineering. I would also like to thank my committee, Dr. Suzanne Paulson, Dr. Stolzenbach, and Dr. Mahendra for guiding me along the way and helping me with all my questions and meanwhile showing me how to be a professional scientist. I would also like to thank my labmates, who have been a great joy to learn from and alongside. I would also like to thank NRDC (Natural Resources Defense Council) and IoES (Institute of the Environment and Sustainability) for the support and finding throughout the multiple projects.

Three chapters in this dissertation are under review. Chapter 2 is a version of the manuscript "Comparison of antibiotic resistance in airborne bacteria near conventional and organic beef production systems in California, USA" currently submitted in *Environmental Science and Technology.* My co-authors are Cristina Echeverria*,* Vanessa Thulsiraj*,* Ariel Flores Madeleine Laitz, Gregory Healy, Shaily Mahendra, Suzanne E. Paulson, Yifang Zhu, and Jennifer A. Jay (PI). Chapter 3 is a version of the manuscript "Antibiotic resistance of *Escherichia coli* isolated from *Conventional, No Antibiotics,* and *Pristine Organic* chicken meat" currently submitted in *Applied and Environmental Microbiology.* My co-authors are Vanessa Thulsiraj, Andrea Maben, Carolyn Collins, Lauren Sevigny, Benjamin Schmidt, Edwin Giragossian, Allison Hornstra, Sarah Kamel, Elizabeth Roswell, Cindy Xiong, and Jennifer A. Jay (PI). Chapter 4 is a version of the manuscript "Airborne Antibiotic Resistance Genes Upwind and Downwind of CAFO Poultry Sites" currently submitted in *Environmental Science and Technology Letters.* My co-authors are Allison Hornstra, Suzanne E. Paulson, and Jennifer A. Jay (PI).

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**Chapter 1: Introduction to the Antibiotic Resistance in Bacteria Isolated from Commercial Meat Samples and Air Samples Near Agricultural Sites**

**1. Introduction**

The World Health Organization (WHO) recently issued its first global report on antibiotic resistance, revealing a serious, worldwide threat to public health [1]. While much attention has been focused on clinical overuse of antibiotics, up to 70% of antibiotics produced in the U.S. during 2008 were used for growth promotion in livestock alone [2]. The common practice of applying sub-therapeutic dosages of antibiotics to livestock has been shown to foster the development of antibiotic resistant bacteria (ARB). [3,4,1,5] ARB originating in livestock can reach the general population via multiple pathways: air downwind of animal feeding operations and transportation vehicles[6,7,8], indoor air[9], soil following land application[10], surface and groundwater[11], and retail meat and poultry. [12, 13] Likewise, antibiotic resistance genes (ARGs) confer antibiotic resistance through various mechanisms, and are themselves considered to be emerging contaminants. [14,15,16]

Bacteria from fecal matter dust inside CAFOs have potential to become airborne and transferred to the outside environment via the large fans used to keep coupes highly ventilated[5]. This contamination of surrounding air downwind of facility has been observed in swine CAFOs[6,7]. The study found the presence of ARB inside the facilities and up to 150 meters downwind of a facility [6]. However, fewer studies have shown the release of ARB specifically from poultry CAFOs.

Confined animal feeding operations (CAFOs) create environments for bacterial populations that inevitably propagate antibiotic resistance within animals and humans. This in

1

turn changes the resistome (collection of ARGs in microbial communities) and allows the antibiotic resistant genes (ARGs) to spread into the surrounding environment. Dutch scientists started monitoring ARGs in agricultural soils as early as the 1940s and have observed an increase in antibiotic resistance gene abundances in soil over time, such as a 15-fold increase of ARGs today compared to the 1970s. The highest rates of increase were shown by tetracyclines (*tet*(Q), *tet*(O), *tet*(M) and the beta-lactamase, *bla*$_{TEM}$ [17]. Common antibiotics used in agriculture include beta-lactamases and macrolides such as erythromycin. The resistance genes associated with these antibiotics have been studied to understand their fate in the soil environment. In Knapp et al. 2010, of eleven ARGs tested in samples from intensive agricultural operations, *erm*(B), *erm*(F), *bla*$_{TEM}$, *bla*$_{SHV}$ were found in abundance in the environment. That finding showed the importance of ARG release to the environment and called for best management strategies and environmental policies related to the handling of agricultural outlets to the environment (Knapp et al 2010). In the Peak (2007) study, ARGs (*tet*(O)), *tet*(Q), *tet*(W), *tet*(M), *tet*(B), *tet* (L), were tested at eight lagoons from CAFOs to understand how feedlot operations affect ARGs in downstream surface waters.[18] This study found that *tet* gene levels were higher in abundance in lagoons that higher usage from farms than lagoons that had no usage.

Another route of exposure is via the retail meat sold in stores. Consumers that purchase poultry products which utilize antibiotics in production may also be affected through consumption and cross-contamination on surfaces. To minimize their exposure, consumers should be adequately informed of antibiotic usage in retail poultry. Thus, consumers may be exposed to antibiotic resistant bacteria (ARB) as a result of farming practices. Similarly, CAFO farm workers and families living on farms using antibiotics in the feed, as well as the neighboring families, had an elevated risk of exposure to antibiotic resistant *E. coli.* [19,20,21]

In Chapter 2, we demonstrate the presence of both ARB and ARGs emanating from cattle production in air. This study is unique in that it is the first to compare resistance in airborne bacteria near conventional and organic beef farms. In Chapter 3, we present results that suggest that the presence of antibiotic resistant *E. coli* differs depending on the type of poultry production system. In this study, we cultured *Escherichia coli* from retail poultry falling into three categories of farming practices: *Conventional, No Antibiotics,* and *Pristine Organic*. In Chapter 4, the study addresses the gap in literature where there are few studies that address the release of ARGs specifically from poultry CAFOs. Regular monitoring and surveillance of ARG from poultry sources is suggested to detect development of airborne antimicrobial resistance that can be spread to surrounding community, especially humans living near farms and workers, via wind air trajectories. The work presented in these chapters can advance regular monitoring and surveillance of ARB and ARG to detect development of airborne antimicrobial resistance that can be spread to surrounding community, especially humans living near farms and workers, via wind air trajectories and retail meat.

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**Chapter 2: Comparison of antibiotic resistance in airborne bacteria near conventional and organic beef production facilities in California, USA**

**Abstract**

Agricultural use of antibiotics selects for antibiotic resistant bacteria (ARB), which may then migrate offsite. Conventional and organic farming practices for cattle, poultry, and swine have previously been compared with respect to ARB from water, soil or fecal samples and information on resistance to antibiotics is variable; moreover, little work has been done to investigate presence of airborne ARB near cattle production farms. In this study, airborne bacteria were collected downwind of three conventional and three organic cattle farms in California, USA. This work tested the hypotheses that: 1) there may be differences in antibiotic resistance to six antibiotics between isolates collected from air near conventional and organic beef cattle farms; and 2) antibiotic resistance genes (ARGs) may be present at differing amounts downwind of organic and conventional farms. We used two methods to assess antibiotic resistance: our newly developed high throughput method (HT) for liquid cultures, and a common method, disk diffusion (DD), which involves culturing on solid media. In this work, many isolates that were not characterized as antibiotic resistant by DD did show resistance when grown in planktonic culture. For HT only, the average fractions of isolates (total n = 1295) resistant to each antibiotic administered at low concentrations on nutrient agar (NA) medium for conventional and organic farms, respectively, were as follows: penicillin: 0.87 and 0.62, cloxicillin: 0.83 and 0.62, oxytetracycline: 0.81 and 0.61, cefoperazone: 0.81 and 0.62, amoxicillin: 0.75 and 0.65, sulfamethazine: 0.82 and 0.65. Of the ARGs tested, either $bla_{TEM}$, $bla_{SHV}$, or $erm$(F) was detected at each of the conventional farms, but not at the organic farm

7

sites. Regular surveillance of ARB and ARGs from beef cattle farms is suggested to detect the spread of ARB and ARG to the community via air trajectories.

**Introduction**

The rising level of antibiotic resistance is a serious public health issue, posing a global threat to human health.[1-3] The agricultural industry is one of the largest consumers of antibiotics.[4-5] The common practice of applying sub-therapeutic dosages of antibiotics to livestock has been shown to foster the development of antibiotic resistant bacteria (ARB).[6,7,1,8] Antibiotic resistance genes (ARGs) confer antibiotic resistance through various mechanisms, and are themselves considered to be emerging contaminants.[9,10,11] ARB originating in livestock can reach the general population via multiple pathways: air downwind of animal feeding operations and transportation vehicles[12,13,14], indoor air[15], soil following land application[16], surface and groundwater[9,17], and retail meat and poultry.[18, 19] To our knowledge there are no previous data on presences of ARGs emanating from cattle production in air.

Previous work has shown an association between the prevalence of ARB in agricultural settings and farming practices. [24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35] Over the past 70 years, food animal production, especially in the U.S., has transformed from small scale to large industrial scale production.[20] Processes in these conventional farming concentrated animal feeding operations (CAFOs) include breeding, feeding, and animal husbandry, all of which may involve antibiotic use for therapeutic and non-therapeutic purposes. In contrast, organic farms within the U.S. are require cattle to be raised without antibiotics or hormones, and feed must be certified as pesticide and antibiotic-free by the U.S. Department of Agriculture.[21,22] Organic farms typically provide

8

more access to free range grazing, sunlight, fresh air, and freedom of movement than their CAFO counterparts.[22]

An important distinction between "conventional" and "organic" farming practices is the known use of antibiotic-enriched feed in the former. Antibiotics are granularly premixed into feed or solubilized into drinking water, aiding in beef cattle weight gain and increased muscle to feed efficiency ratio[23]. Another popular feed for domestic livestock is distillers' grain solubles, a byproduct of ethanol production from corn. Antibiotics including penicillin, erythromycin, tylosin, and virginiamycin are added in the production of distillers' grain solubles to prevent overgrowth of bacteria in ethanol grain reactors. There is some evidence that drying inactivates the antibiotics, but no data are available on ARB and ARG in this feed.[24]

Conventional and organic farming practices, for cattle[25-28], poultry[29-33], and swine[34-36], have previously been compared with respect to ARB and information on resistance to antibiotics is variable. In general, increased resistance has been shown in dairy, poultry and swine[24-35] but not for beef. Few studies have investigated beef cattle; the majority have focused on the dairy industries. For dairy, Sato et al.[26] found that *Escherichia. coli* isolates from conventional dairies had significantly higher resistant rates than isolates from organic dairies. Cho et al 2007[27] studied dairy cattle from both conventional and organic farms and found a significant difference in STEC (Shiga toxin–producing *Escherichia coli*) resistant to spectinomycin (72% conventional and 39% organic). A study from Halbert et al.[27] showed mixed results and observed that in *Campylobacter* isolates from dairy cattle farms of both production types, were mostly susceptible; however, resistance was slightly higher in conventional isolates than organic for tetracycline. However, for beef cattle, ARB patterns have shown no increased resistance for

conventional farming. Reinstein et al.[25] reported no major difference between organic and conventional isolates from fecal samples.

While previous studies have measured ARB from CAFOs[12,13,15], few have focused on offsite migration of ARGs However, there is some information regarding bacteria emanating from swine facilities. Bacteria near and inside swine CAFO facilities can become airborne, contaminating surrounding air downwind of the facility and transporting ARB offsite.[13] Our study is unique in that it is the first to compare resistance in airborne bacteria near conventional and organic beef farms.

However, there are no previous data on presence of ARGs emanating from the two types of cattle food production, conventional and organic. One study by Knapp et al 2010[38] found the abundance of ARGs increased in agricultural soil from the 1940's to the present, with the highest rates of increase for resistance to tetracycline $tet$(Q), $tet$(O), $tet$(M) and beta-lactams, $bla_{TEM}$. Another study found the presence of ARGs in river sites coming from CAFO's and wastewater treatment plants.[39] Despite these investigations into soil and water, few studies have researched into the connection between air transport and ARGs from CAFOs. La Para et al 2013[40] suggested ARG transport from CAFO's and clinics; $tet$(W) genes were discovered within 2 km of CAFO site. Thus, presence of ARG in air adjacent to cattle animal farming operations and organic operations and how it compares in relevance to antibiotic resistance warrants further investigation.

Although cattle farms are prevalent throughout California, the impact of farming practice (conventional or organic) on levels of ARB and ARGs in local air has not been documented. This work (n=1295 isolates) tested the hypotheses that: 1) there may be differences in antibiotic resistance to six antibiotics between bacteria isolates collected from air near conventional and

organic beef cattle farms; and 2) ARGs may be present at differing amounts downwind of organic and conventional farms. This study expands upon current monitoring approaches by combining molecular techniques and two culture-based methods to determine presence of ARB and ARGs in air samples. Molecular assays were optimized for quantification of ARGs from air samples. Both the standard disk diffusion method, which involves growth on Petri dishes, and a newly developed high-throughput antibiotic resistance screening method involving liquid culture, were employed to assess resistance.

**Materials and Methods**

Sample site and collection

The three conventional beef cattle production sites, recognized as feedlots, were in Kern and Fresno Counties, located in the Central Valley of CA, a highly intensive agricultural region. The three organic production sites were located in Santa Barbara and Ventura Counties. We were not able to locate an organic site in close proximity to the conventional sites.

Six sampling events occurred over the summer of 2013. Sampling events between a conventional and an organic farm were conducted on consecutive days to minimize effects of weather variability. All sampling took place on dates with clear, dry weather and moderate winds. Sample collection occurred within 3 meters of the edge of the farm; animals were visibly present near the perimeter of each farm when sample collection took place. (A more detailed table with information for each farm is provided, Table S1)  Also, sampling was performed at locations where odor was prominent.

The first week of sampling events for conventional (C) and organic (O) farms, respectively, took place on June 25 2013 (C1) and June 28 2013 (O1). The second week consisted of two field events on July 2 2013 (C2) and July 3 2013 (O2), and the third week

samples were collected on July 11 2013 (O3) and July 12 2013 (C3). Sampling occurred for each farm between the hours of 11:00 am and 5:30 pm. At each farm, a single integrated sample was collected at 12.5 liters per minute (lpm) for four hours for ARB analysis and triplicate samples were collected at 2 lpm for four hours for ARG analysis.

Samples were collected for the purpose of capturing ARB, using a bioaerosol sampler (SKC BioSampler, SKC Inc. Eighty Four, PA.) with a 15 mL liquid medium of 10% glycerol and 90% water. A vacuum pump (SKC Inc. Eighty Four, PA. Cat No. 228-9605) was used to draw in air and was connected to a car battery for power. The flow rate of 12.5 lpm was initially set controlled before collection and checked periodically. The bioaerosol sampler was placed adjacent to the farm site (within 3 meters distance) and collected air for approximately four hours. After the four-hour sampling period, the collection liquid was transferred to a 50 ml falcon tube (Fisherbrand) and transported to the laboratory on ice for sample processing for bacterial purification and antibiotic resistance testing.

For ARG assays, samples were collected in triplicate for four hours on 47 mm diameter glass fiber filters (EMD Millipore Glass Fiber Filters without Binders, Catalog No: APFA 047 00) for the purpose of capturing air-borne bacteria for DNA extraction and analysis of ARG. Air was sampled at three locations near the farm and effort was made to be as close as possible to farm without entering private property. Samples analyzed for ARGs were collected from conventional and organic sites within 3 meters distance from the edge of the farm. Air samples were collected using personal pumps (SKC 224-PCXR4 Aircheck Sampler, SKC Inc. Eighty Four, PA) at a flow rate of 2 lpm for approximately four hours, after which filters were placed in petri dishes and transported to lab on ice. Once in the lab, filters were stored at -80°C up to seven months until further processing for molecular analysis of ARG.

Bacterial Identification

16S rDNA identification was tested on a subset of PCA and of NA to compare similarities in microbial communities among farm sites sampled. 17 isolates of PCA were analyzed from each of the two production types: conventional and organic. 11 isolates of NA were analyzed from each of the two production types: conventional and organic. At least five isolates from each farm were selected. A previously established DNA extraction protocol (Shanks et al. 2012)[41] was used to extract DNA from all isolates. Afterwards, polymerase chain reaction (PCR) was used to amplify the 16S rDNA of the isolates, followed by a purification step with the MoBio 12500-50 UltraClean PCR Clean-Up kit. Gel electrophoresis was used on a selection of samples to verify good quality of PCR product. In addition, DNA quantity and quality was assessed using UV absorption with a Nanodrop 2000C (Thermo Scientific, Waltham MA). Further processing and sequencing of the 16S gene was performed at UCLA Genotyping and Sequencing Core (GenoSeq, Los Angeles, CA). Sequences were then blasted using NCBI Blasting website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the bacteria.

Culture work for antibiotic resistant bacteria

Isolates were cultured from the Bioaerosol liquid sample collected from each site. For each site, 50 μL of the sample was spread onto Petri plates containing 20 mL of either nutrient agar (NA) or plate count agar (PCA). Two types of media were used to culture a diverse bacterial population. A total of 10 NA and 10 PCA plates were prepared for each farm site. Inoculated plates were incubated for 48 hours at 25$^{\circ}$C. After two days, a subset of randomly selected bacterial colonies (obtained from proportional allocation) were streaked onto new media plates three times consecutively to purify.

Total number of bacterial isolates cultured and tested were at least 100 for each of the plate types (NA and PCA) from each of the six farms. Thus, a total n of 688 NA isolates from conventional farms and total n of 607 isolates from organic farms were analyzed for antibiotic resistance. The total of 1295 (688 and 607) purified isolates were inoculated into a microcentrifuge tube consisting of 2 ml of medium broth (NA or PCA) and grown overnight at $25^{o}$C while continuously shaking on a Rotor Shaker at level 3.

Antibiotic resistance testing

The high-throughput antibiotic resistance screening tested 1295 bacterial isolates for resistance using a newly developed antibiotic resistance test that compares growth in planktonic culture, which is distinct from the standard disk diffusion method. For this assay, a 1:40 dilution of isolate into medium broth was used when dispensing into high-throughput 384 well plates (E&K Scientific). Our assay (SI Figure 1) tested six antibiotics at two concentrations. By looking at the EUCAST database (distribution graphs from http://www.eucast.org/mic_distributions/) for each species, we chose the highest MIC breakpoint to be our lowest concentration level. We then chose a second higher concentration to test. The six antibiotics selected for analysis were: penicillin (2048 mg/L, 1024 mg/L), oxytetracycline (8 mg/L, 2 mg/L), cefoperazone (512 mg/L, 128 mg/L), amoxicillin (256 mg/L, 64 mg/L), cloxicillin (512 mg/L, 128 mg/L), sulfamethazine (2048 mg/L, 1024 mg/L) (Sigma-Aldrich).

A no antibiotic control was included for each isolate on each 384 well plate. Absorbance readings of plates at OD 600 at 0, 4, and 24 hours were obtained by using the GloMax® Microplate Multimode Reader (Promega). Resistance ratios were obtained by comparing control

isolate growth rates to isolates with antimicrobial growth rates. Resistance ratio cutoff was 0.4 for all isolates tested.

The standard method of measuring for antibiotic resistance, disk diffusion, was also used to test 100 isolates (50 conventional and 50 organic) in triplicate. In this method, each bacterial isolate (50 μL) was uniformly spread onto the surface of a Mueller-Hinton agar (MHA) plate with an antiseptic metal spreader to form an even film. Antibiotic paper disks (6 mm in diameter; BD Diagnostic Systems) were placed on the surface of each seeded MHA plate using a sterile pair of forceps. Plates were incubated at 35°C for 16-18 hours. During incubation, antibiotic agents diffuse outwards, creating regions of inhibition within the microbial lawn. The diameters (mm) of the zones of inhibition were measured by a ruler or caliper. Based on the diameter of the inhibition zone and the CLSI interpretative criteria, the isolates are typically assigned to three categories: susceptible, intermediate, or resistant. For this study, the intermediate and susceptible categories were grouped. The smaller the diameter of the inhibition zone, the more resistant is the microorganism to the antibiotic.

Molecular work for antibiotic resistant genes

DNA was extracted from 47 mm glass fiber filters using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc, Carlsbad, CA, Catalog No. 12888-50).[42] DNA processing was optimized for the filters used in this study. While polycarbonate filters are traditionally used for molecular based work, a short experiment resulted in comparable DNA yield between polycarbonate and glass fiber filters. The process was also optimized based on extraction kit, and MoBio Power Soil and Gene Rite DNA extraction kits were both found to have comparable performance in terms of both DNA quantity and quality across both filter types. Glass fiber

filters were selected for use because the SKC 224-PCXR4 Aircheck Sampler used in this experiment were intended for this particular filter type. Modifications on the DNA extraction protocol were made accordingly. First, the filters were sliced in half using sterile scalpels, folded and aseptically added into separate extraction beaded tubes containing buffer, and agitated for 2 minutes. A comparison of DNA sample extraction using whole filter versus half was conducted to determine best yield (data not shown). Each filter half was extracted independently and run through the same spin column in the final stages of the established MoBio protocol, yielding a 100μl total volume which was stored at -20°C for further analysis with quantitative polymerase chain reaction (qPCR).

qPCR Methods

The abundances of ARGs were measured using qPCR (Applied Biosystems) (Table S2). ARGs, $bla_{TEM}$, $bla_{SHV}$, $erm$(F), $erm$(B), $tet$(M), $tet$(O), $tet$(W), $tet$(L), $van$A, $van$B were chosen because they have high prevalence in environmental samples.[38, 42] The ARGs of interest required both TaqMan and SYBR-green Master Mix kits to target genes. For genes requiring SYBR, we used a reaction mixture of 25 uL volume containing 12.5 μL of 1X SYBR® Green Master Mix (Applied Biosystems), 0.25 ng of the template DNA, 1.25 μL (see Table S2 for final concentrations) of forward and reverse primers and 8 μL molecular grade $H_2O$. For other genes, we used Taqman Master Mix (Applied Biosystems), with a reaction volume of 25 μL; containing 12.5 μL of 1X SYBR® Green Master Mix (Applied Biosystems), 2μL of the DNA samples, 1.25 μL of both forward and reverse primers, 1 μL of Taqman probe, and 7 μL molecular grade $H_2O$. Reactions were then performed in triplicate using the ABI 7700 Sequence Detection System. Table S2 lists the primers and reaction conditions used in the study. Melting curve analysis was conducted to

indicate correct positive amplification of target gene. Samples from each farm site were analyzed in triplicate and the threshold cycle ($C_T$) value was averaged for each sample. A positive detection for each sample was defined as at least two of three replicate wells showing amplification with a $C_T$ value less than 40.

**Results**

Sample site and collection

At three of the conventional sites (C1, C2, C3) and 2 organic sites (O2, O3), wind was weak for at least part of the time, and direction was variable. Odor was prominent in sites C1, C2, and C3. Under these conditions, plume spread over short distances to a degree (50 m) in all directions is anticipated. At the one sites (O1) with steady wind (>5 mph for whole sampling period), the sampling sites were downwind more than 70% of the time. Conventional sites have higher temperatures than organic sites. Two of the conventional sites (C2 and C3) had very low relative humidity; one site (C1) was well within range with the other organic sites (Table S1).


Bacterial Identification

A subset of isolates (n=71) cultured on either PCA or NA from conventional and organic sites was identified using 16S rRNA gene sequencing. For the PCA organic isolates (n=17), the following was found in the subset: 41% *Bacillus*, 35% *Enterobacter*, 12% *Staphylococcus*, 6% *Massilia*, and 6% *Clavibacter*. For PCA conventional isolates (n=28), the following was found in the subset: 36% *Bacillus*, 29% *Enterobacter*, and 29% *Staphylococcus, 3% Paenibacillus and 3% Lactobacillus*. For the NA organic isolates (n = 11), the following was found in the subset: 55% *Bacillus*, 36% *Enterobacter*, and 9% *Ornithinibacillus*.

17

<u>Antibiotic resistance of isolates</u>

A total of 1295 bacterial isolates from locations adjacent to six farms (n > 200 for each farm) were cultured and were tested for antimicrobial resistance. In general, conventional beef cattle production locations consistently showed a greater percentage of the total bacterial isolates resistant to antibiotics tested compared to organic production (Figures 1A, 1B). For NA medium, the average fractions of resistance ratios (of three farms) for conventional and organic, respectively, for each antibiotic (low concentrations) were determined to be as follows: penicillin: 0.87 and 0.62, cloxicillin: 0.83 and 0.62, oxytetracycline: 0.81 and 0.61, cefoperazone: 0.81 and 0.62, amoxicillin: 0.75 and 0.65, sulfamethazine: 0.82 and 0.65 (Figure 1A). A Welch t-test was done on conventional verse organic for each antibiotic for NA medium. Penicillin ($p<0.02$), cloxicillin ($p<0.03$), oxytetracycline ($p<0.02$), and sulfamethazine ($p<0.05$) showed significant differences between resistance ratios for conventional and organic sites. Results for high antibiotic concentrations also showed generally higher fraction of isolates resistant, significant for penicillin ($p<0.00$), cloxicillin ($p<0.02$), and sulfamethazine ($p<0.05$) (Figure 1B).

For PCA medium, the average fraction (of three farms) for conventional and organic, respectively, for each antibiotic (low concentrations) were determined to be as follows: penicillin: 0.78 and 0.62, cloxicillin: 0.83 and 0.62, oxytetracycline: 0.88 and 0.73, cefoperazone: 0.90 and 0.60, amoxicillin: 0.84 and 0.65, sulfamethazine: 0.85 and 0.69 (Figure S2A). A Welch t-test was done on conventional verse organic for each antibiotic for PCA medium. Oxytetracycline ($p<0.04$) showed a significant difference between resistance ratios for conventional and organic sites at the low concentration. At high concentrations, the pattern is similar but not statistically significant for any antibiotic.

18

Consistently, conventional production sites showed a greater average fraction of ARB than organic production for all six antibiotics at the low and high concentrations. At least one antibiotic from each antibiotic class tested showed more resistance in conventional farming practices than in organic. Penicillin and cloxicillin (penicillin group), oxytetracycline (tetracyclines), cefoperazone (cephalosporins) and sulfamethazine (sulfonamides) showed greater resistance in conventional farming. Isolates exposed to higher antibiotic concentration showed the same antimicrobial resistance pattern but showed more variability in the conventional farms.

A subset of isolates was analyzed both by the high-throughput method, which involves growth in liquid culture, as well as the standard disk diffusion method, which assesses resistance on a solid media (Figure 2, Figures S3-S25). Thus, resistance in both biofilm and planktonic growth modes can be assessed. SI graphs show scatter plots of the results from both tests. These graphs can be divided into four quadrants. Isolates characterized resistant by the HT and DD methods are represented on the top half, and the right half, of the plots, respectively. Isolates characterized as resistant in both planktonic and biofilm growth modes fall in the top right quadrant. Notably, the upper right quadrant shows isolates that would be susceptible by DD but are resistant as characterized by HT. Data from the scatter plots are also presented as bar charts comparing the fraction of resistant isolates from organic and conventional farms by HT, DD, and by both methods.

Several patterns emerged from the analysis by dual methods. First, in the case of penicillin, the fractions of resistant NA isolates were not different between conventional and organic by the HT method. However, DD did show a more prominent increase in resistance at conventional facilities (Figure S3 illustrates Pattern 1). This was also observed in other cases

19

(cloxicillin (NA), sulfamethazine (NA), cefoperazone (NA), amoxicillin (PCA)), with the additional result that isolates resistant by both methods were also higher in conventional farms (Pattern 2: Figure 2, Figures S6-S12).   A third pattern is illustrated in Figures S13 and S14 (Pattern 3).  In the case of amoxicillin and cefoperazone (PCA, higher dose) DD did not show a difference between organic and conventional, but analysis in planktonic culture by HT did show a much more pronounced difference between farm types.

qPCR for ARGs

Bioaerosol samples collected within three meters distance from the perimeter of each farm were analyzed for multiple ARGs. Each of the three conventional farms showed presence of at least one ARG in proximity to the site, as follows: C-1: $bla_{TEM}$, C-2: $bla_{SHV}$, C-3: $erm$(F). No organic site showed any presence of ARG (Table 1). Neither conventional nor organic sites showed any presence of $erm$(B), $tet$(M), $tet$(O), $tet$(W), $tet$(L), $van$A, or $van$B.

**Discussion**

Conventional beef production sites showed a greater average fraction of ARB than organic production for most of the six antibiotics at the low and high concentrations, some with statistical significance (Figure 1, Figure S2). As tested by the HT method, antibiotic resistance in airborne isolates was significantly greater for conventional isolates for at least one antibiotic from each class of antibiotics tested: the $\beta$-lactams group (penicillin, amoxicillin, cloxicillin, and cefoperazone), the tetracycline group (oxytetracycline), and the sulfonamides (sulfamethazine).

A subset of isolates was tested by both HT and DD methods, and several patterns emerged.  The first and second pattern showed some antibiotics from the beta-lactam group, such

as penicillin and cloxicillin showing a somewhat higher level of resistance in the conventional isolates by HT, and a more marked difference by DD. $\beta$-lactam antibiotics act by inhibiting synthesis of the peptidoglycan layer of bacterial cell walls., and are useful against a broad spectrum of pathogens, including *E. coli* and *Klebsiella pneumonia*. Sulfamethazine (in NA at high concentration) also fell in the second pattern, where isolates resistant by both methods were marked increased at conventional facilities relative to organic. Sulfamethazine, a sulfonamides, which consist of sulfamethazine and sulfathiazole, are analogs of para-aminobenzoic acid (PABA), inhibiting DNA synthesis by disrupting normal utilization of PABA. A third pattern is illustrated in Figure S13, where analysis by disk diffusion shows a difference between organic and conventional, but analysis in planktonic culture by HT did show a much more pronounced difference between farm types. We found this to be true for amoxicillin (at high concentration in PCA). It has been suggested that chromosomally encoded drug-deactivating enzymes may collect in the biofilm matrix and decrease the effect of $\beta$-lactam antibiotics.[43,44,45] Thus this could suggest why there is no pronounced distinction in DD.

Our study focused on two types of growth conditions: biofilm (DD) and planktonic cells (HT). When analyzing by both methods, in some cases two different antibiotics acting with similar mechanism have different effects for biofilm and planktonic. All patterns were observed (A, B, C): A) disk diffusion would show the antibiotic (sulfamethazine, oxytetracycline, amoxicillin, cloxicillin, penicillin (NB)) as more effective in biofilm B) high-throughput would show more efficacy of antibiotic (amoxicillin, cloxicillin, penicillin (PCA)) in planktonic. C) One case of showed comparable results (cloxicillin). Notably also, many isolates fall in quadrant 2, which means they would be categorized as susceptible by DD but are resistant in planktonic culture.

Many studies reported that biofilms are more antibiotic resistant than planktonic cells.[46,47,48,49] When comparing resistance between biofilm culture and planktonic cultures, there is typically a 10-1,000 fold difference in susceptibility. Biofilm culture are known to have a compact nature with extracellular polymetric surfaces separated by water channels ensuring the diffusion of oxygen, nutrients, and waste product. They have reduced rates of cellular growth and respiration; thus, attack and destruction of the biofilm is less likely than in their counterpart of planktonic cultures.[49] Also, another factor of biofilm tolerance is the presence of unknown resistance mechanisms. [50]

While it is generally thought that biofilms having greater resistance than planktonic cells, one study claimed that this premise was not justified since biofilm cultures depend largely on persister cells.[51] Their study suggests that persisters, cells that persist living but with a disabled PCD mechanism, is what allows for planktonic cells to have the advantage in antibiotic resistance over biofilm cells. Other studies concluded that persister accumulation can occur if mutations happen increasing the number of persisters in exponential phase in *E. coli*. [52, 53, 54]

Resch et al 2004 [55] demonstrated through side by side planktonic and biofilm testing that the gene expression patterns are distinct under the two growth conditions. Biofilm cells had their cell envelope as the most active compartment: cell wall synthesis and function were up-regulated. Conversely, planktonic cells had toxins and proteases up-regulated.

However, in our study for the HT method, the planktonic cells were suspended in nutrient broth or plate count broth. This may explain why the mechanisms of resistance associated with a protective matrix was diminished. Many bacteria that are present in air are not culturable at present. This was our reasoning to use NA and PCA in order to capture as much of the population as possible. Even though selective media can pinpoint any potential pathogens, by

using NA and PCA we are also able to do the same since normal heterotrophic bacteria in air can potentially carry resistance.

These results are in accord with previous comparisons of antibiotic resistance between organic and conventional farming. Sato et al.[26] stated that *E. coli* isolates from conventional dairies had significantly higher resistant rates than isolates from organic dairies for the following antibiotics: ampicillin, streptomycin, kanamycin, gentamicin, chloramphenicol, tetracycline, and sulfamethoxazole. Gebreyes et al.[34] showed resistance for tetracycline and erythromycin for intensive confined farming higher than free range farming in *Campylobacter* isolates, which is not surprising as oxytetracycline and sulfamethazine are both antibiotics used commonly in cattle feed in conventional farming in the United States.[23] In our study both oxytetracycline and sulfamethazine showed a significant higher fraction of isolates resistant in conventional production than organic production. Reinstein et al.[25] found no major difference between organic and conventional isolates from beef cattle.

Additionally, ARG were observed at measurable levels within proximity to conventional farms, but not organic farms. Chosen genes of $bla_{TEM}$, $bla_{SHV,}$ $erm$(F) have been observed in environmental samples previously: in surface waters[56], recycled water[57], agricultural sites[38, 42, 58] and river sediments.[59, 60] Ling et al. 2013 [40] found similar results when comparing CAFO's and clinics to a homeless shelter and found that CAFOs had 10 to 100 times higher concentrations of airborne 16S rRNA, *tet*(X), and *tet*(W) genes than the other sites. In addition, they conclude that CAFOS are possible sources of ARGs and can transported locally via the air pathway.

This study expands upon current monitoring approaches by combining both culture-based methods and molecular techniques to determine presence of ARB and ARGs from airborne bacteria. Molecular assays were optimized for detection of ARGs from air samples. This new sampling approach provides a unique framework for future studies that aim to test for presence of airborne ARB.

Both results suggest that the use of antimicrobials on farms can influence the bacterial resistance patterns depending on beef production type, conventional or organic farming. Surveillance at both farm production types are needed but it is especially advised for conventional farm practices.

World Health Organization (2014)[2] stated "a need for an improved and coordinated global effort, including wider sharing of surveillance data". Only a limited number of countries have surveillance programs for production animals such as Canada, Denmark, Germany, Japan, Netherlands, and the U.S. Recently, in December 2013, the United States FDA proposed out a voluntary road map plan for industry[61] to revise and phase out certain antibiotics from food animal production. Whereas the U.S. holds less stringent policies, other countries like Denmark have held stronger regulations in place to monitor antibiotic resistance. The Center for Disease Control's report 2013[62] remarks also that the link between antibiotic use in animal production and AR infections in humans calls for use of antibiotic use under veterinary oversight, not for growth promotion.

There are several routes by which animal agricultural use (AAU) can contribute to higher occurrence of ARB within and outside of agricultural settings. Livestock given antibiotics can foster the emergence of new resistant strains of bacteria. In the gut bacteria of the animal, ARG operons can be accumulated on integrons and transferred to plasmids and other moveable

24

elements. AAU selects for assembly of resistance gene clusters that then move to commensals and pathogens in the microbial community by horizontal gene transfer (HGT). HGT occurs between strains of the same species and between species via several mechanisms including plasmid transfer, phage transduction, and transformation. The result is a larger reservoir of ARB than occurs in the absence of selective pressure by antibiotics, and these ARB serve as vectors that move ARG to human microflora.

In contrast, ARG can also be transferred to commensal and pathogenic bacteria that are capable of human-to-human transmission. A recent study showed evidence for transmission of ARGs from environmental bacteria to pathogens.[63] Some bacteria, such as vancomycin-resistant enterococci can serve as pathogens in both animals and humans, and once they cross the species barriers can result in sustained illness in humans.[64] However, even non-pathogenic, commensal bacteria originating in animals are a source of ARG in the human microbiome that may subsequently be passed among species.

There are several routes by which humans may be exposed to ARB and ARG originating in AAU. Occupational exposures have been documented[65,66] and the potential impacts of ambient exposure to ARG and ARB are not currently understood. ARGs have been measured in air, sediments, surface waters, and drinking waters, although there is still a paucity of data in this area.

The high throughput may be a useful addition to current tools in antibiotic resistance testing. The high-throughput antibiotic resistance screening method: 1) allows for analysis of 12 isolates at two levels of an antibiotic in one assay 2) can obtain results in as little as 4 hours (depending on growth rate of organisms and media used) 3) can test multiple mediums to capture

greater diversity of microbial community 4) allows for analysis of six different antibiotics at two concentrations in one assay.

The higher frequency of both ARB and ARG detection near conventional versus organic farms collectively show the occurrence of antibiotic resistance in the environment, which may imply potential exposure to ARB and ARGs via an air pathway. Regular monitoring and surveillance of ARB from cattle sources is suggested to detect development of airborne antimicrobial resistance that can be spread to surrounding community, especially humans living and working near farms, via wind air trajectories. In this study, it can be suggested that the general microbiome in the conventional area can be impacted by multiple farms and future research can investigate this possible phenomenon further. However, a limitation of this study is that production sites could differ in ARB and ARG because of possible differences in animal population density between sites; however, this difference between groups was unavoidable as it is inherent in the farming types. Most of our results are based on a fraction of total isolate; thus, if so, this points out that production types are better to be small scale than large scale in order to limit the amount of ARB and ARG in the air. Also, if the organic farms were not located in the same general area as the conventional farms, the patterns could purely be climactic, or owing to some other industry in the area. Future research should involve more in depth analysis of mechanisms of antibiotic resistance which is critical for developing any future effective plan for reducing the possible impact of ARB and ARGs on public and environmental safety.

**Table 2 - 1.** ARG presence at Conventional and Organic Site. Any value less than 40 was considered as a positive detection, with at least two of the three wells showing as a positive.

X: no presence; ✓: presence detected

| Type of Farm | $bla_{TEM}$ At Site | $bla_{SHV}$ At Site | $erm$(F) At Site |
|---|---|---|---|
| Organic 1 | X | X | X |
| Organic 2 | X | X | X |
| Organic 3 | X | X | X |
| Conventional 1 | ✓ | X | X |
| Conventional 2 | X | ✓ | X |
| Conventional 3 | X | X | ✓ |
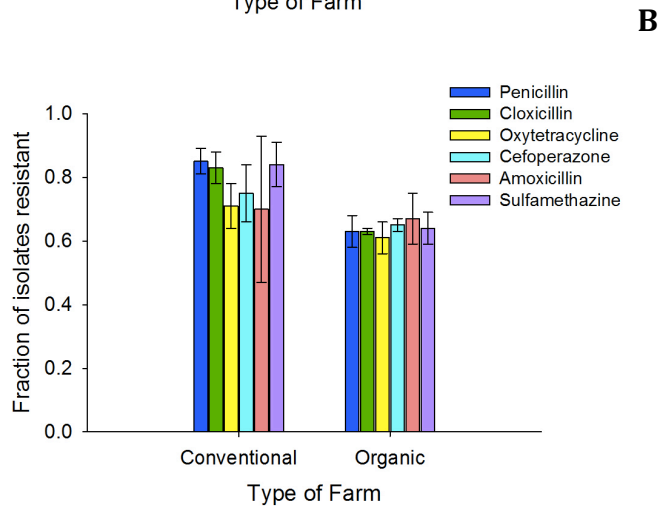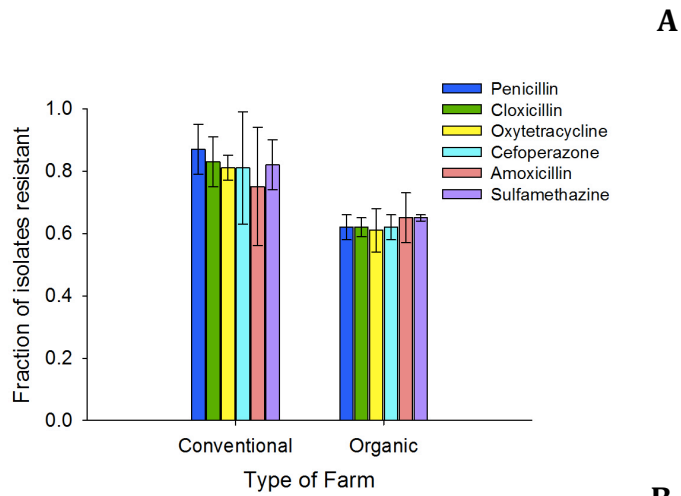
**Figure 2 – 1.** A) The resistance ratios for isolates for designated antibiotics for NA plates (low concentrations) The bars indicate standard deviation. Two-tailed t-test of unequal variance $p < 0.05$, with p values of: penicillin: 0.02, cloxicillin: 0.03, oxytetracycline: 0.02, cefoperazone: 0.19, amoxicillin: 0.49, sulfamethazine: 0.05 B) The resistance ratios for isolates for designated antibiotics for NA plates (high concentrations) The bars indicate standard deviation. Two-tailed t-test of unequal variance $p < 0.05$: penicillin: 0.00, cloxicillin: 0.02, oxytetracycline: 0.14, cefoperazone: 0.16, amoxicillin: 0.84, sulfamethazine: 0.05

CLOXICILLIN LOW NA
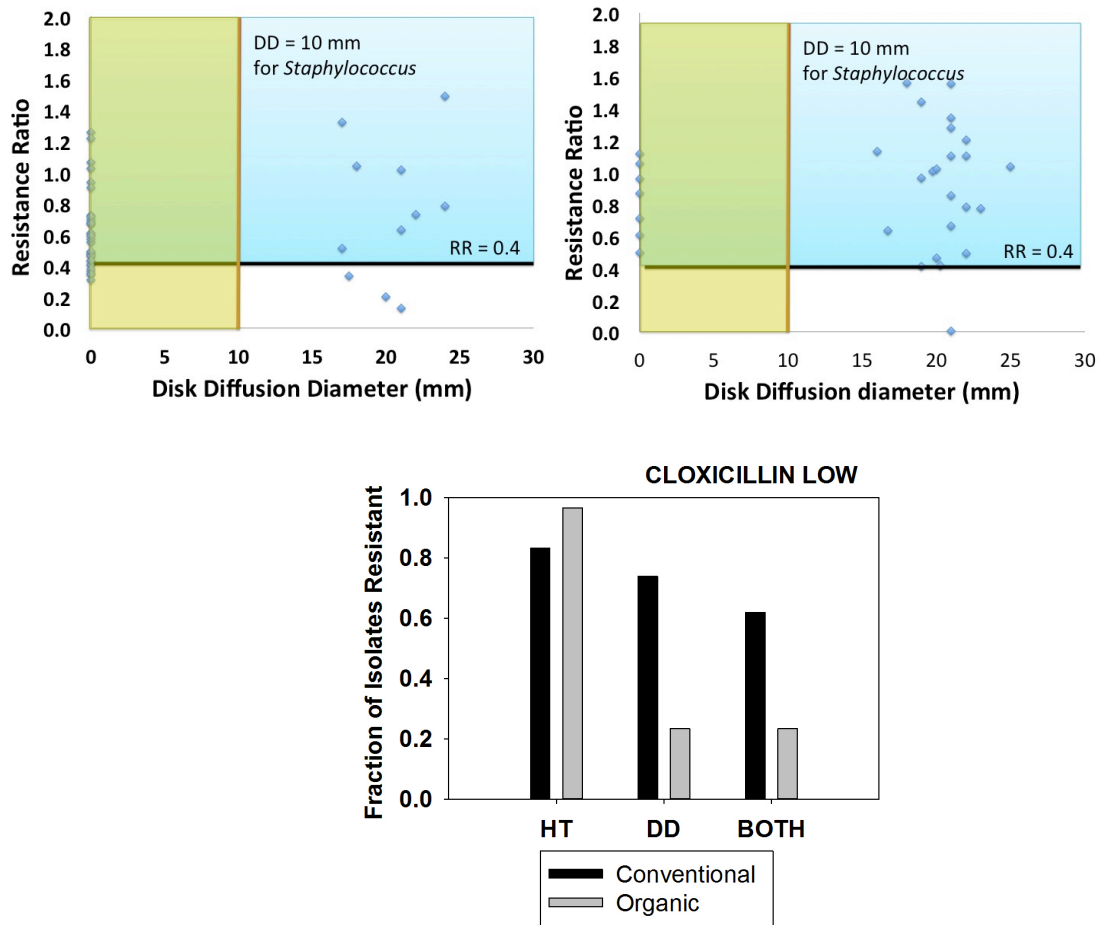


**Figure 2 - 2.** Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods. Cloxicillin at low antibiotic concentration using NA medium. Top graphs show HT methods (left: conventional, right: organic). The black horizontal line defines the resistance ratio line =0.4 and the red vertical line defines the level at which resistance is defined for disk diffusion for that antibiotic for that species. Bottom graph shows comparison of both HT and DD.

**Appendix A**

**Table 2 - 2.** Meteorological data for conventional sites (C1, C2, C3) and organic sites (O1, O2, O3)

| Loc. | Date | Wind Speed (MPH), Wind Direction | T (C°) range | RH (%) range | Notes |
|------|------|----------------------------------|--------------|--------------|-------|
| O1 | 6/28 | 6 – 15, WNW-NE | 30 – 32 | 30-25 | Perfect wind direction for sampling |
| O2 | 7/3 | 0 – 13, 0 – 4 WSW-SW-,SWW; brief stints of WNW-NE sites agree | 20.5 - 22 31-32 | 83 – 70 45-36 | Location is between 2 met stations with steep gradient. Some periods with no wind recorded, which means variable/wind speed too low. |
| O3 | 7/11 | 0 – 11, 113-315° (ESE, S, SW, WSW, WNW, NW) | 24 – 32 | 58 – 39 | Location is between 2 cities, but at same elevation. |
| C1 | 6/25 | 0 - 5, S-E-N; mostly ESE° | 26 – 33 | 60 – 37 | Weak winds, some zero |
| C2 | 7/2 | 0 - 6 0 + WNW - NNE | 39 - 42 | 17 - 13 | Station is near center of valley, slightly south of monitoring sites. |
| C3 | 7/12 | 0 – 6 0 + SSW, NW - NE | 28 - 37 | 20 - 15 | Station is near center of valley, slightly south of monitoring sites. |

**Table 2 - 3.** The primer sequences and qPCR reaction conditions used in the study

| Target | Primer | Concentration | Sequence (5'-3') | Annealing Conditions | Elongation conditions | Reference |
|---|---|---|---|---|---|---|
| $erm$ **(F)** | ErmF-F | 500 nM | TCG TTT TAC GGG TCA GCA CTT | 60°C/30s | | 38 |
| | ErmF-R | 500 nM | CAA CCA AAG CTG TGT CGT TT | | | |
| $bla_{TEM}$ | BlaTEM-F | 400 nM | TCG GGG AAA TGT GCG | 50°C/60s | 72°C/60s | 38 |
| | BlaTEM-R | 400 nM | GGA ATA AGG GCG ACA | | | |
| $bla_{SHV}$ | BlaSHV-F | 400 nM | TGA TTT ATC TGC GGG ATA CG | 55°C/60s | 76°C/30s | 38 |
| | BlaSHV-R | 400 nM | TTA GCG TTG CCA GTG CTC G | | | |
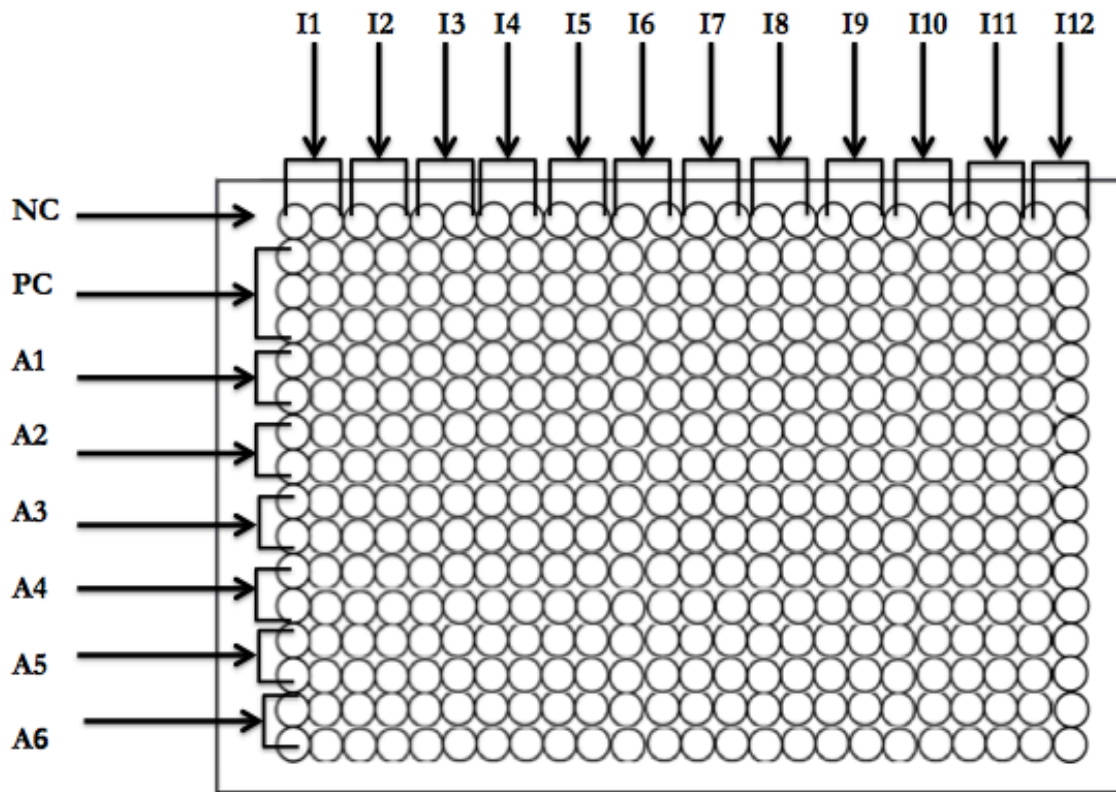
**Figure 2 – 3.** Schematic illustration of the high-throughput method on 384 well plate. NC is negative control for one row consisting of only medium (either NB or PCB); PC is positive control for 3 rows consisting of only medium and isolate; A1-A6 are antibiotics one through six, with each antibiotic having two rows (low and high) consisting of medium and antibiotic.
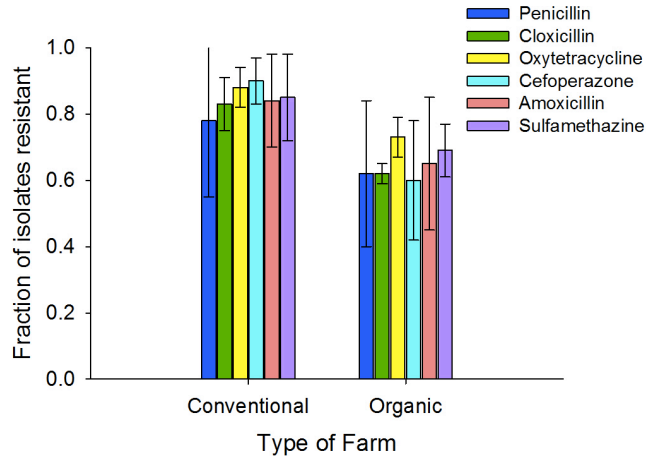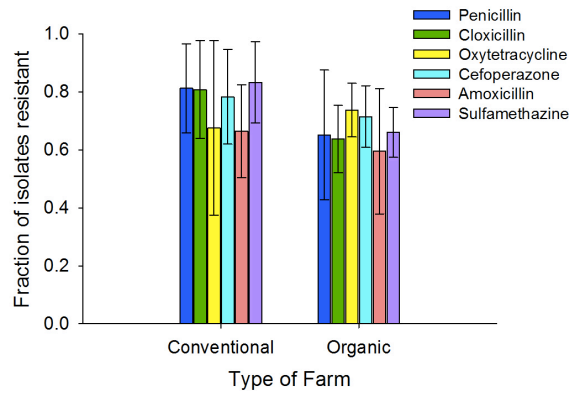
**Figure 2 – 4.** A) The resistance ratios for isolates for designated antibiotics for PCA plates (low concentration) The bars indicate standard deviation. Two-tailed t-test of unequal variance $p < 0.05$ with p values of: penicillin: 0.42, cloxicillin: 0.22, oxytetracycline: 0.04, cefoperazone: 0.08, amoxicillin: 0.27, sulfamethazine: 0.16. B) The resistance ratios for isolates for designated antibiotics for plate count agar plates (high concentration) The bars indicate standard deviation. Two-tailed t-test of unequal variance $p < 0.05$ with p values of: penicillin: 0.37, cloxicillin: 0.23, oxytetracycline: 0.76, cefoperazone: 0.58, amoxicillin: 0.68, sulfamethazine: 0.15.

**For all following figures:**

Top graphs show HT methods (left: conventional, right: organic). The black horizontal line defines the resistance ratio (RR) line = 0.4 and the red vertical line defines the level at which resistance is defined for disk diffusion for that antibiotic for that species. Bottom graph shows comparison of both HT and DD.

Each figure specifies a pattern, which are the following:

- Pattern 1: DD shows an increase in resistance at conventional facilities.

- Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods.

- Pattern 3: HT showed a pronounced difference between farm types than DD.

- Pattern 4: Both DD and HT show consistent difference among farm facilities.

- Pattern 5: No difference among farm facilities.

- Pattern 6: Organic facilities show higher resistance than conventional facilities.
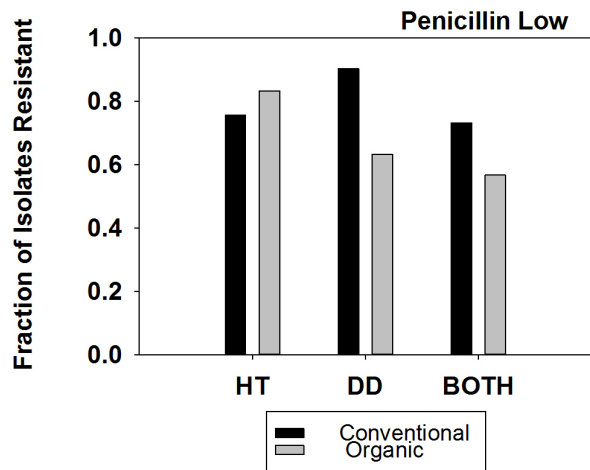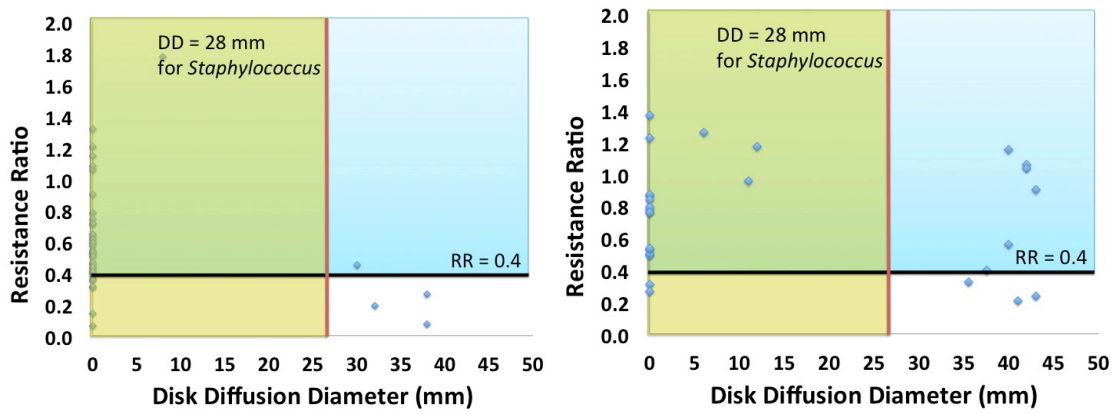
**Figure 2 – 5.** Pattern 1: DD shows an increase in resistance at conventional facilities. Penicillin at low antibiotic concentration using NA medium.
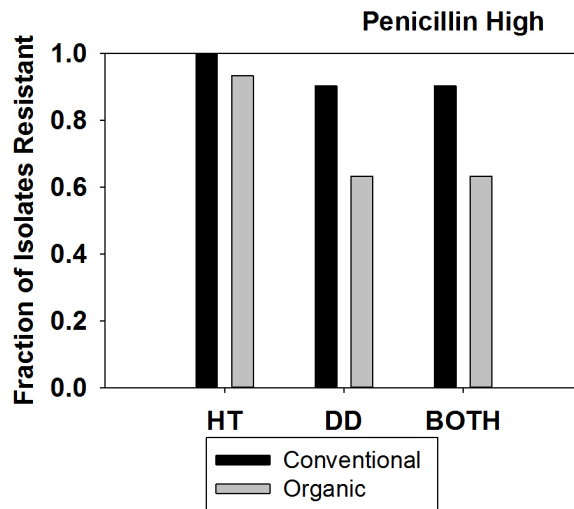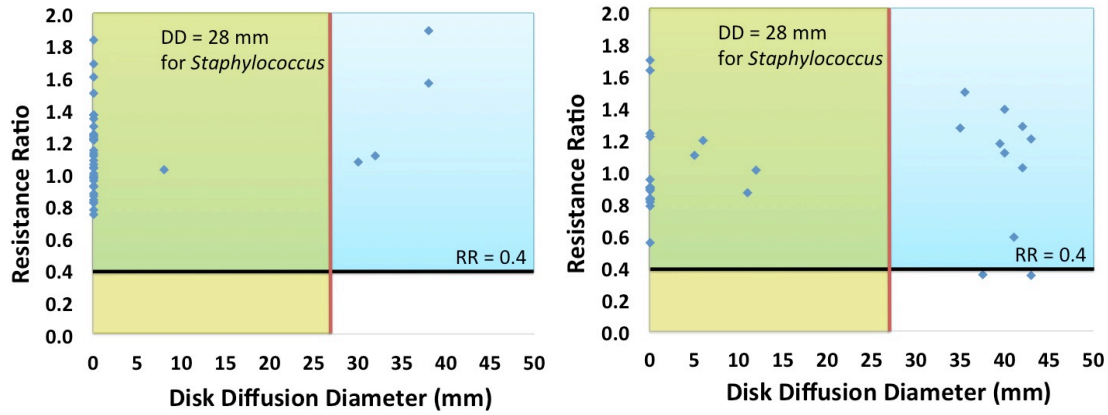
**Figure 2 – 6.** Pattern 1: DD shows an increase in resistance at conventional facilities. Penicillin at high antibiotic concentration using NA medium.
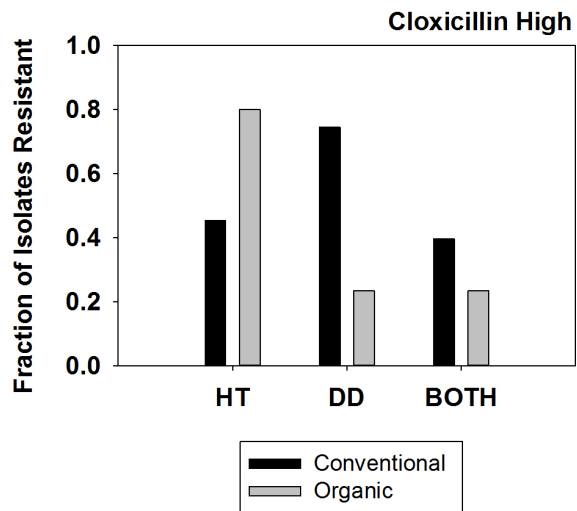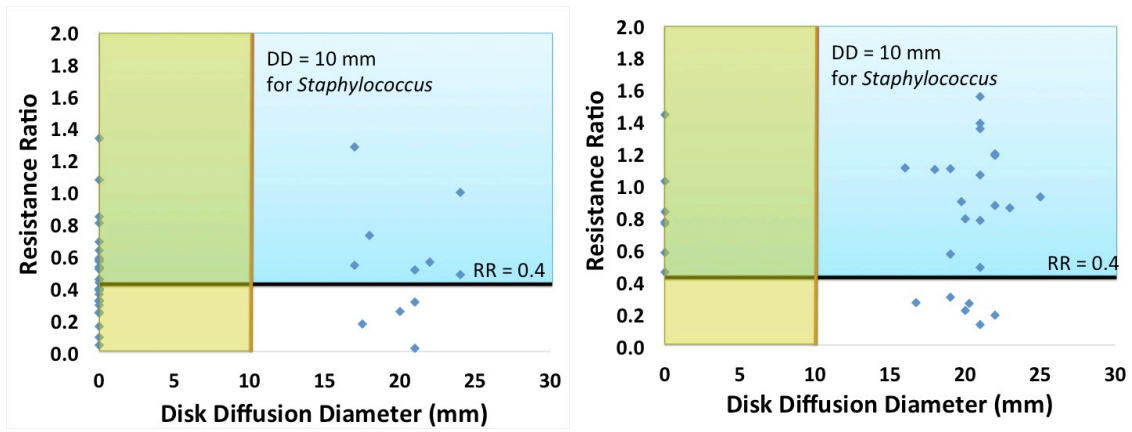
**Figure 2 – 7.** Pattern 1: DD shows an increase in resistance at conventional facilities. Cloxicillin at high antibiotic concentration using NA medium.
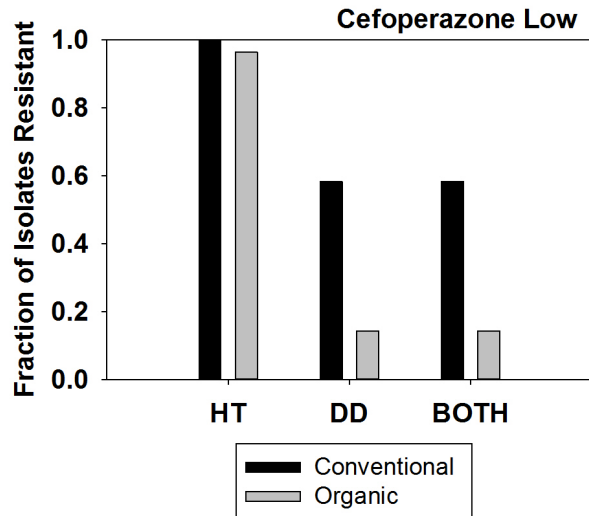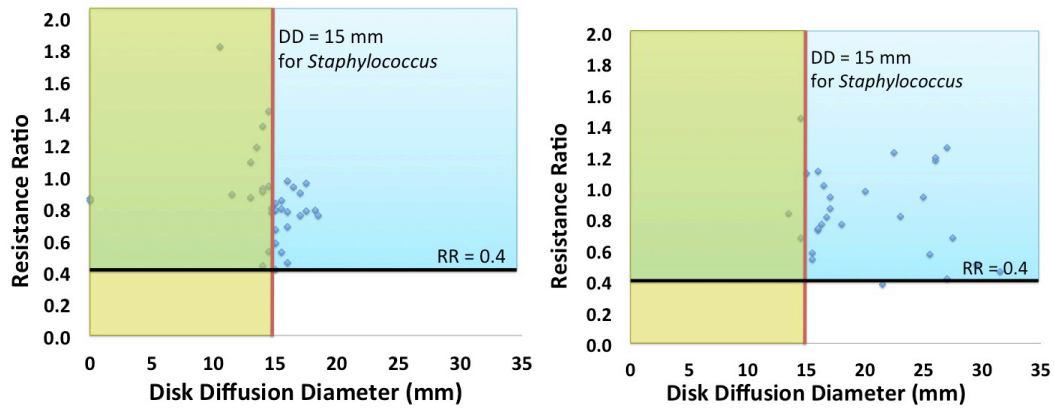
**Figure 2 – 8.** Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods. Cefoperazone at low antibiotic concentration using NA medium.
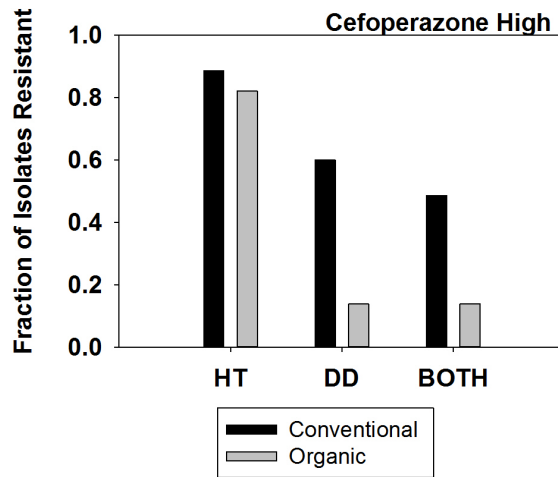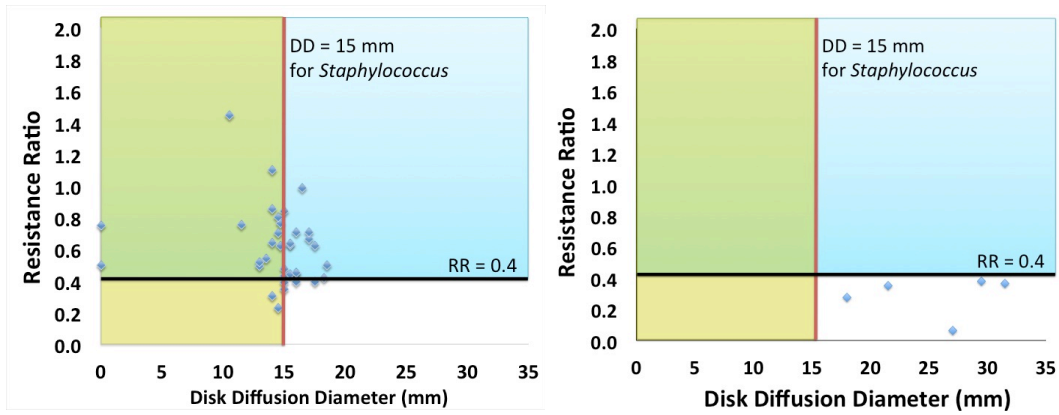
**Figure 2 – 9.** Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods. Cefoperazone at high antibiotic concentration using NA medium.
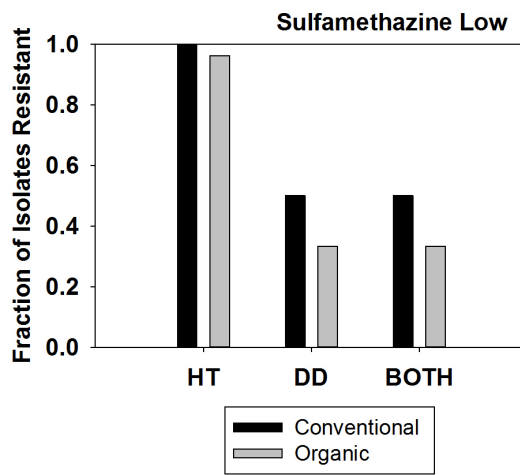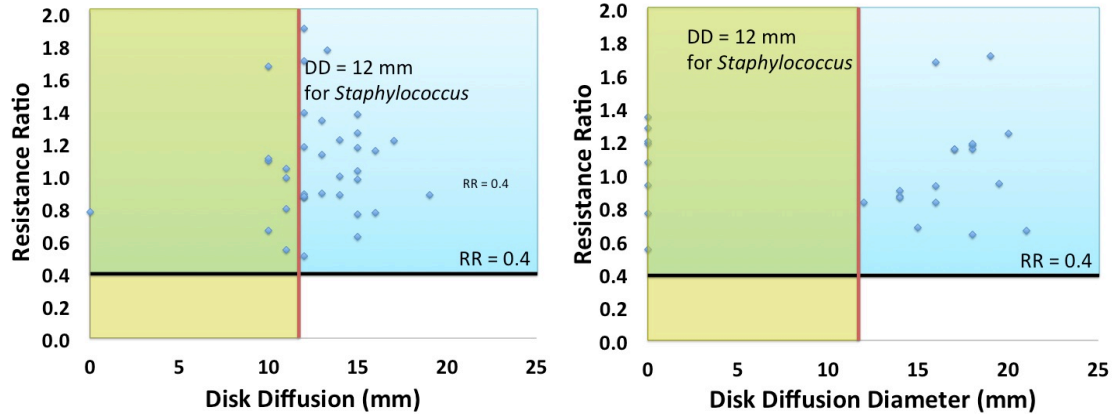
**Figure 2 – 10.** Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods. Sulfamethazine at low antibiotic concentration using NA medium.

**Figure 2 – 11.** Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods. Sulfamethazine at high antibiotic concentration using NA medium.

**Figure 2 – 12.** Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods. Amoxicillin at low antibiotic concentration using PCA medium.
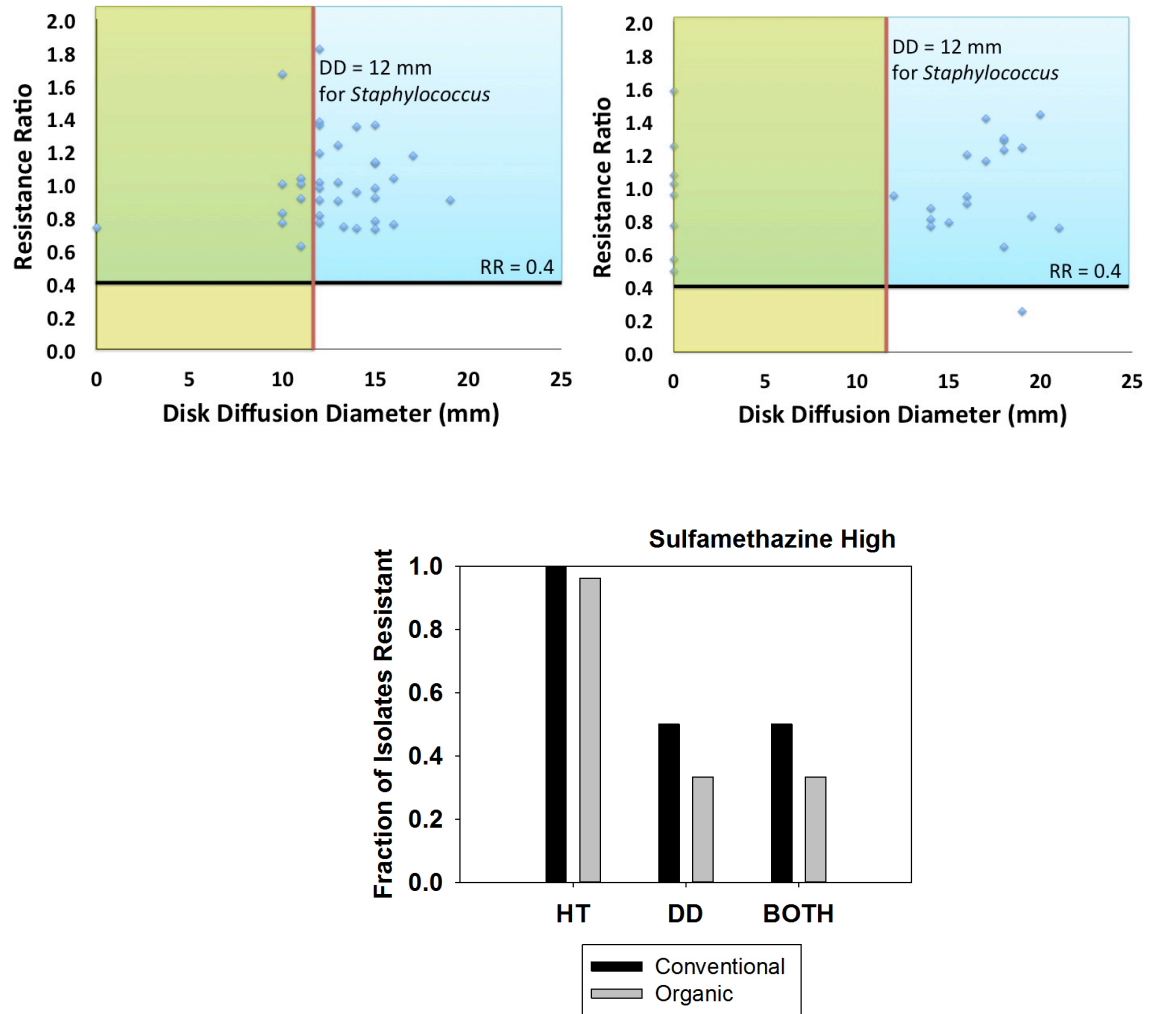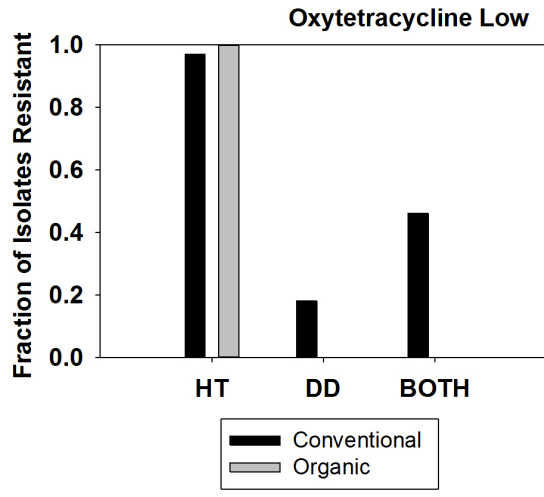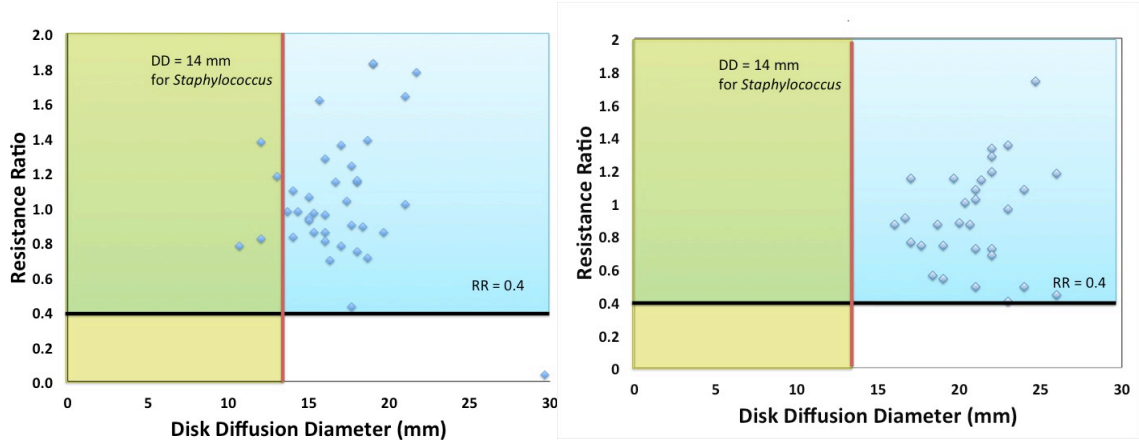
**Figure 2 – 13.** Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods. Oxytetracycline at low antibiotic concentration using NA medium.
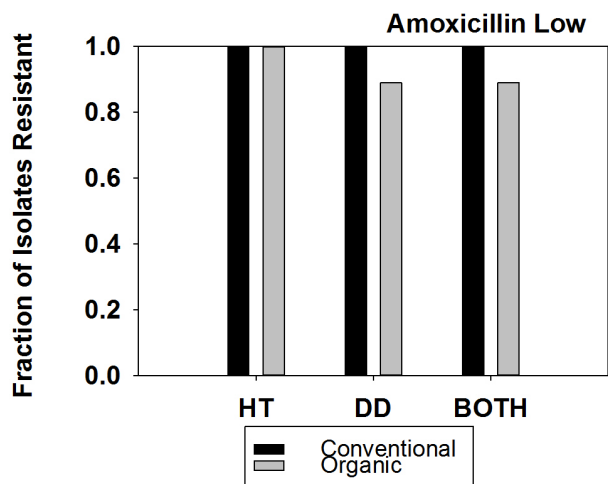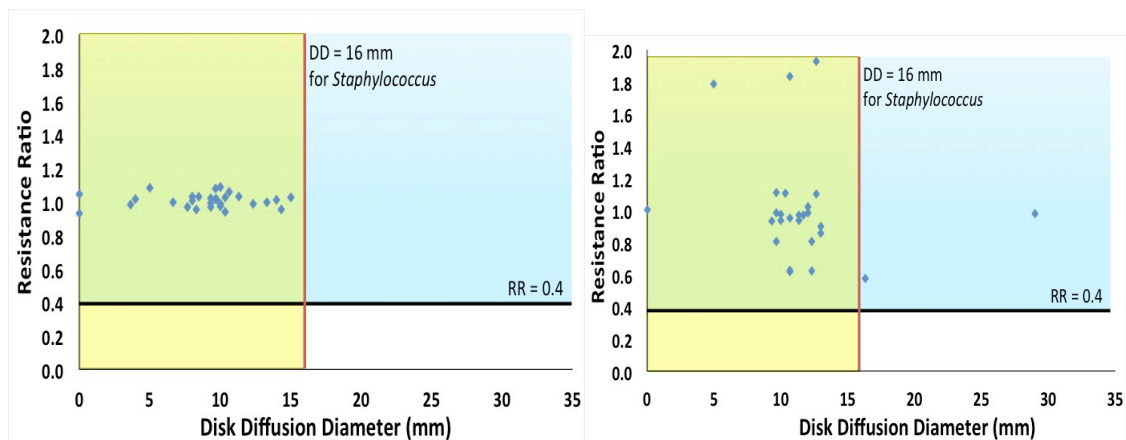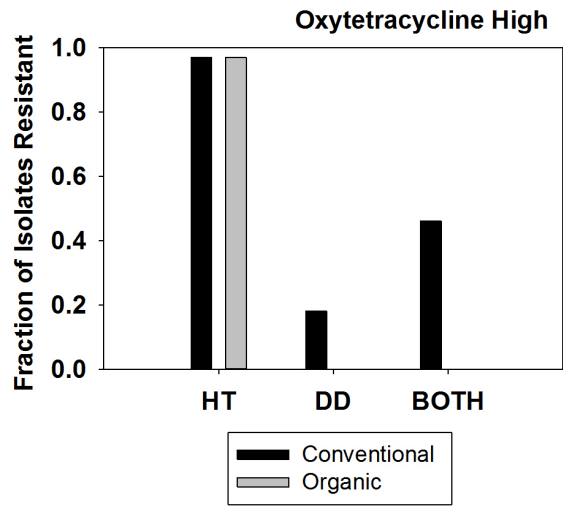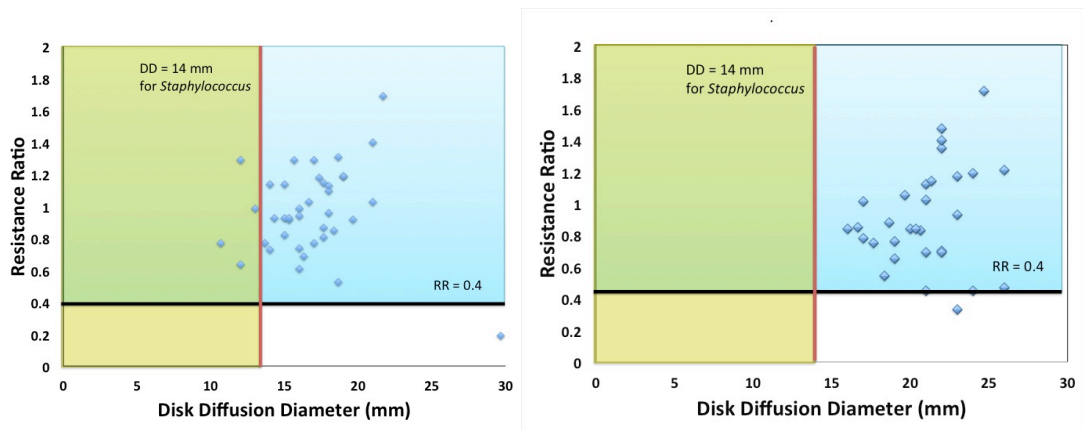
**Figure 2 – 14.** Pattern 2: DD shows an increase in resistance at conventional facilities, with the additional result that isolates resistant by both methods. Oxytetracycline at high antibiotic concentration using NA medium.
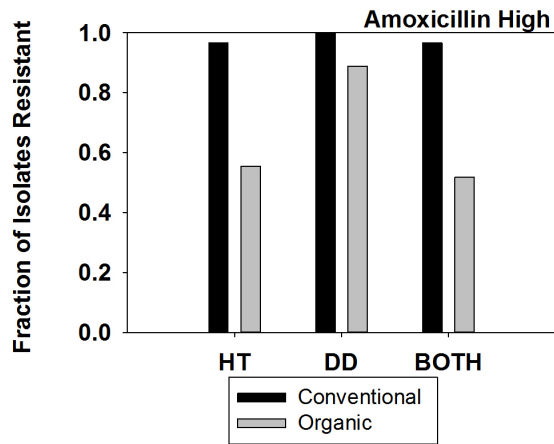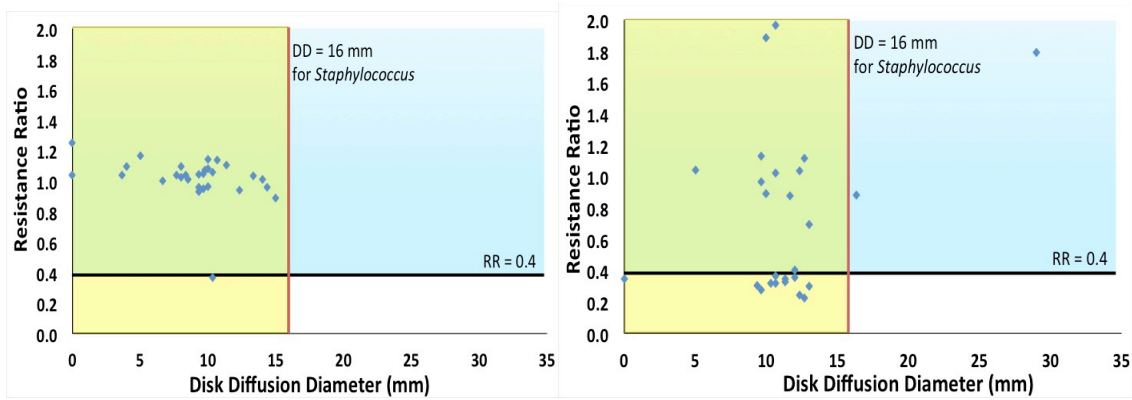
**Figure 2 – 15.** Pattern 3: HT showed a pronounced difference between farm types than DD. Amoxicillin at high antibiotic concentration using PCA medium.
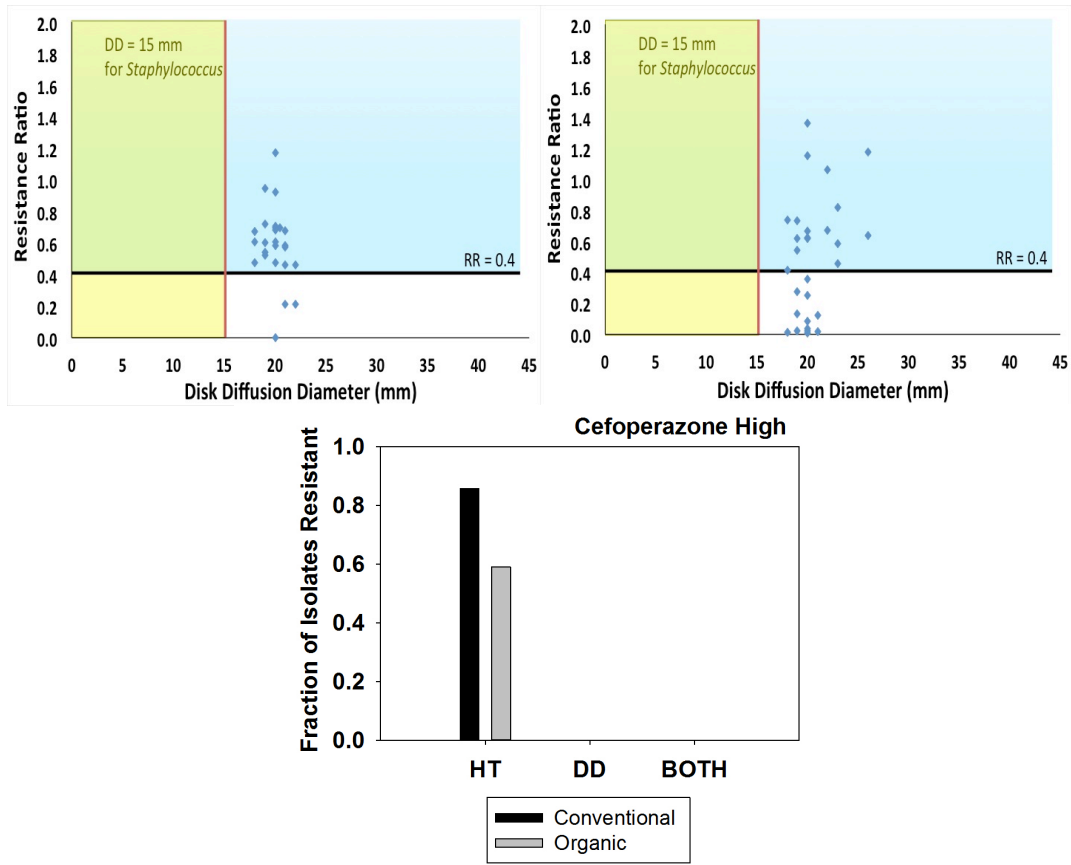
**Figure 2 – 16.** Pattern 3: HT showed a pronounced difference between farm facilities than DD. Cefoperazone at high antibiotic concentration using PCA medium.
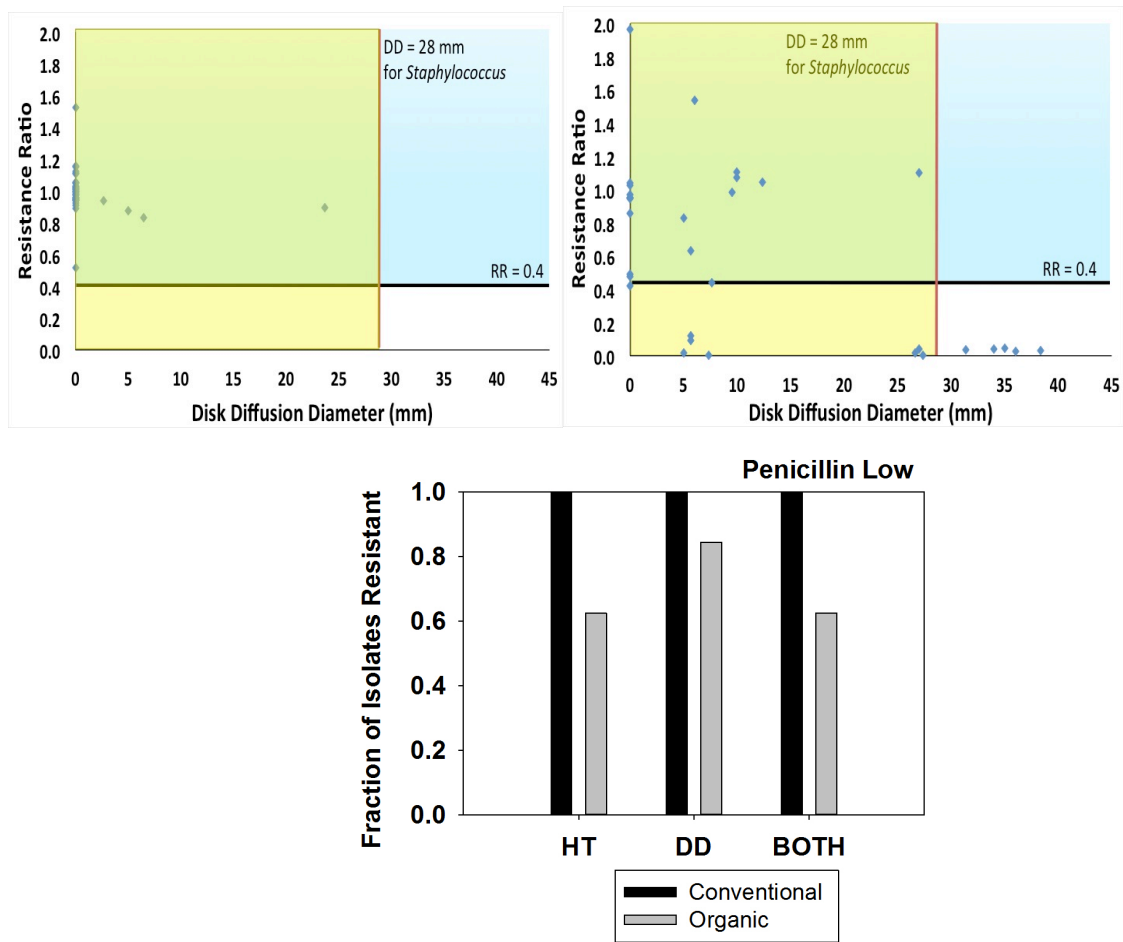
**Figure 2 – 17.** Pattern 4: Both DD and HT show consistent difference among farm facilities.

Penicillin at low antibiotic concentration using PCA medium.
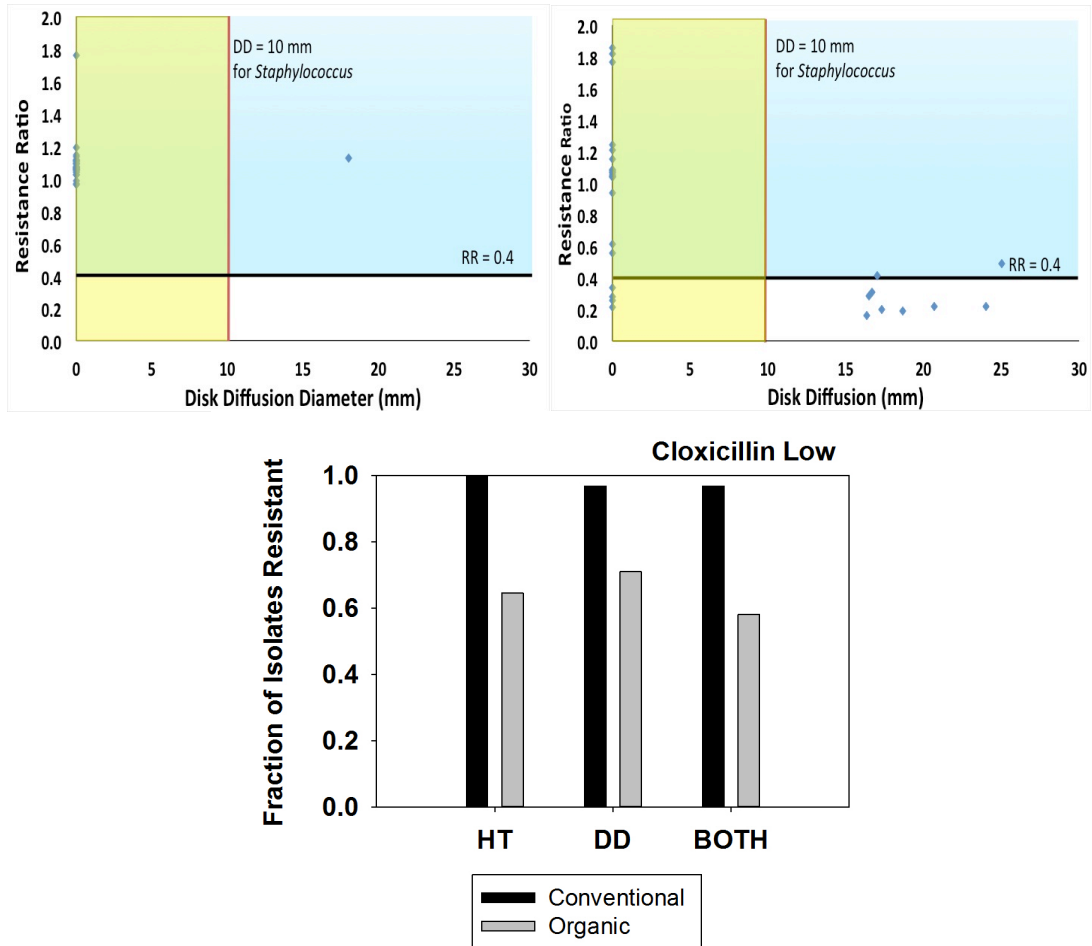
**Figure 2 – 18.** Pattern 4: Both DD and HT show consistent difference among farm facilities. Cloxicillin at low antibiotic concentration using PCA medium.
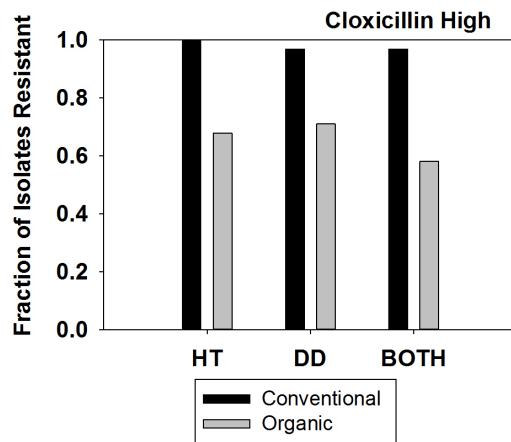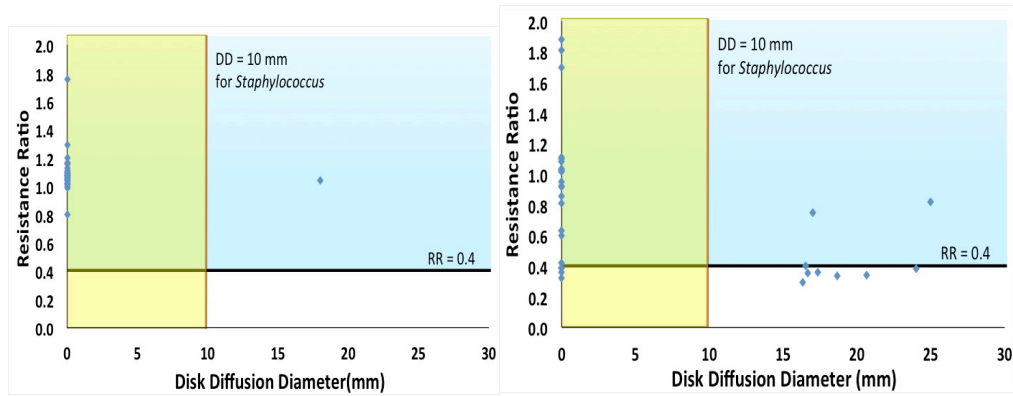
**Figure 2 – 19.** Pattern 4: Both DD and HT show consistent difference among farm facilities. Cloxicillin at high antibiotic concentration using PCA medium.

**Figure 2 – 20.** Pattern 5: No difference among farm facilities. Sulfamethazine at low antibiotic concentration using PCA medium.

**Figure 2 – 21.** Pattern 5: No difference among farm facilities. Sulfamethazine at high antibiotic

concentration using PCA medium.
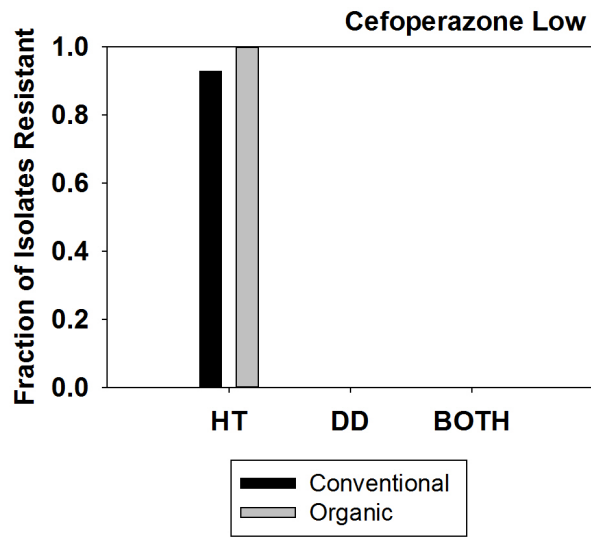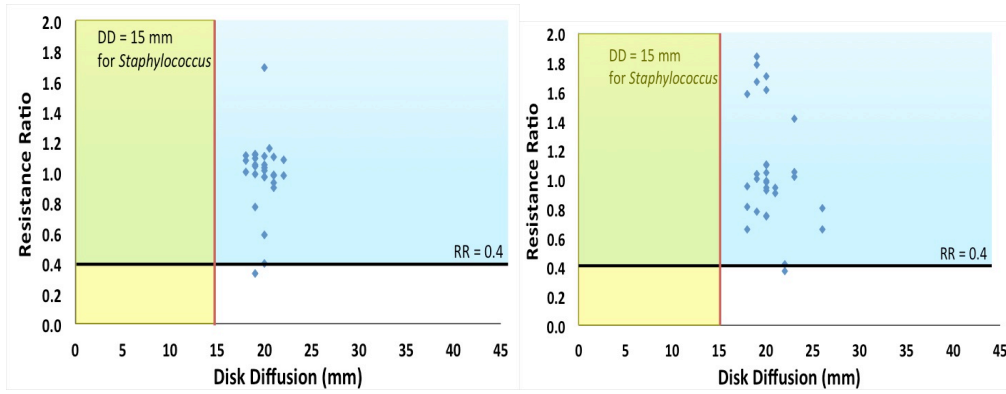
**Figure 2 – 22.** Pattern 5: No difference among farm facilities. Cefoperazone at low antibiotic concentration using PCA medium.
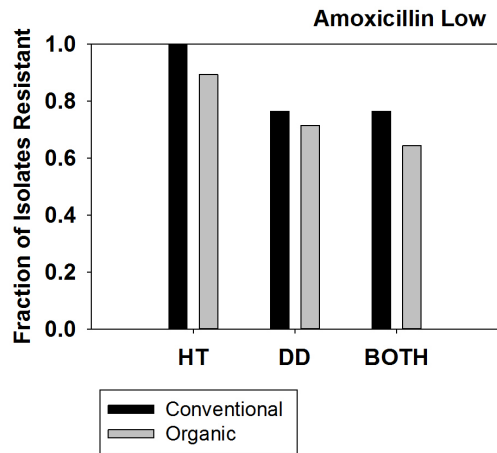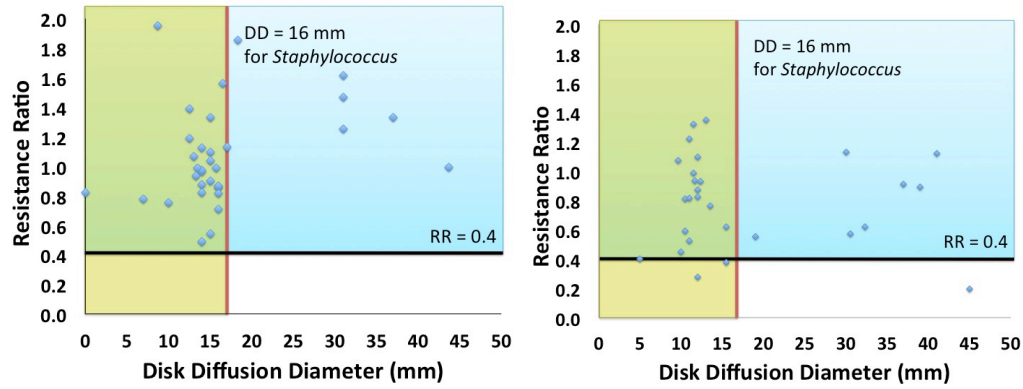
**Figure 2 – 23.** Pattern 5: No difference among farm facilities. Amoxicillin at low antibiotic concentration using NA medium.
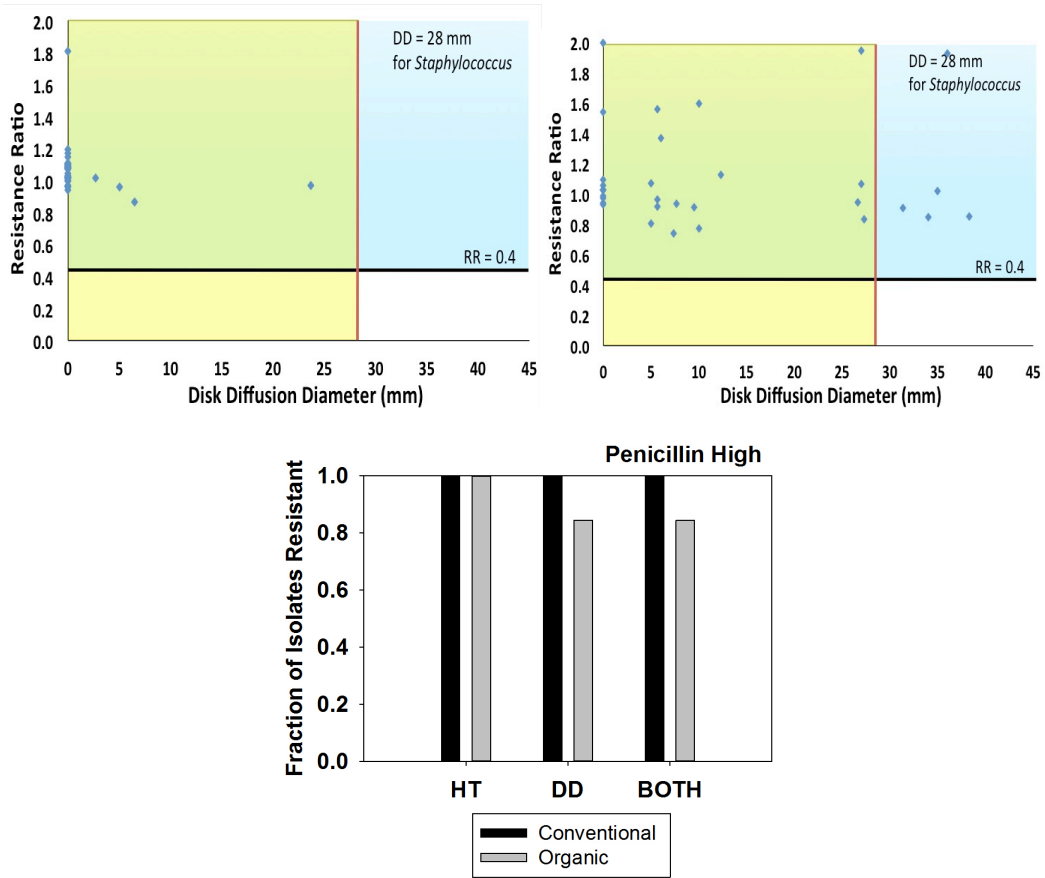
**Figure 2 – 24.** Pattern 5: No difference among farm facilities. Penicillin at high antibiotic concentration using PCA medium.
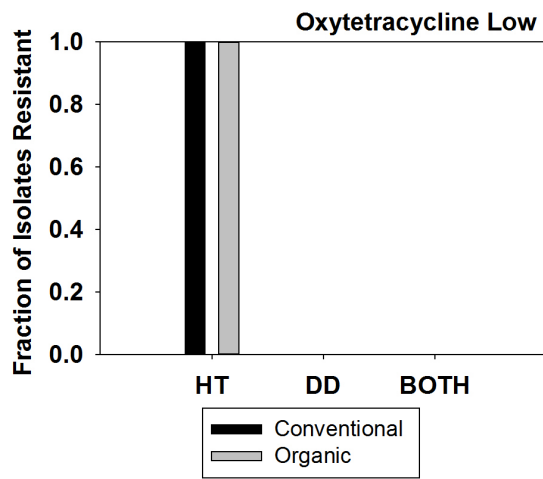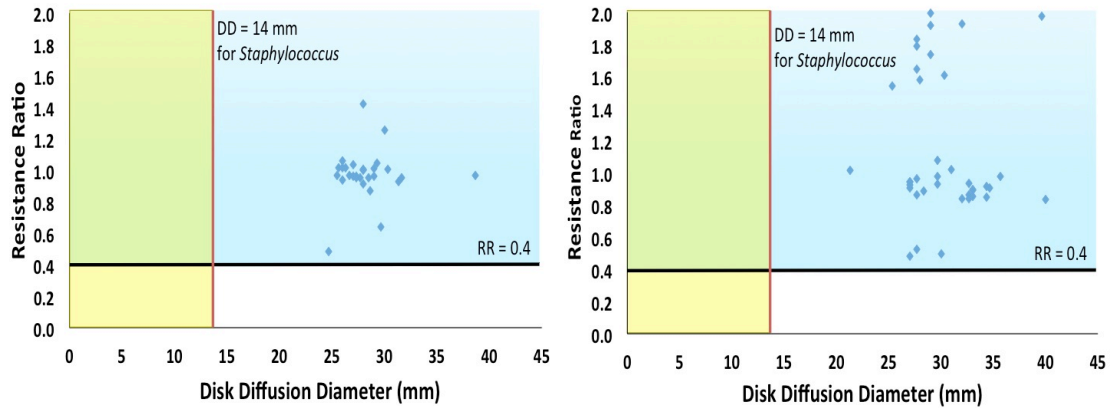
**Figure 2 – 26.** Pattern 5: No difference among farm facilities. Oxytetracycline at low antibiotic concentration using PCA medium.

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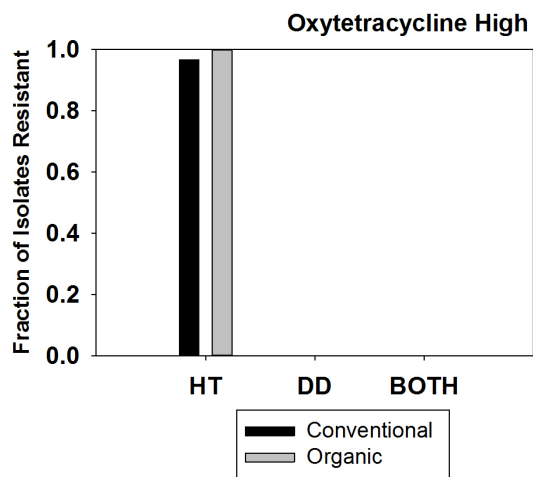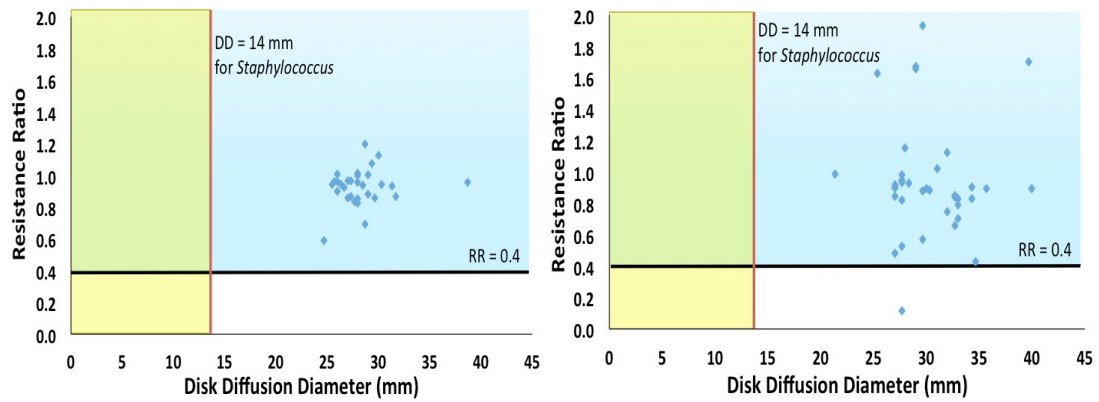
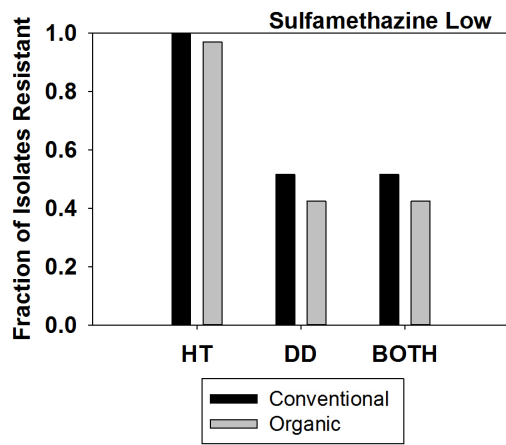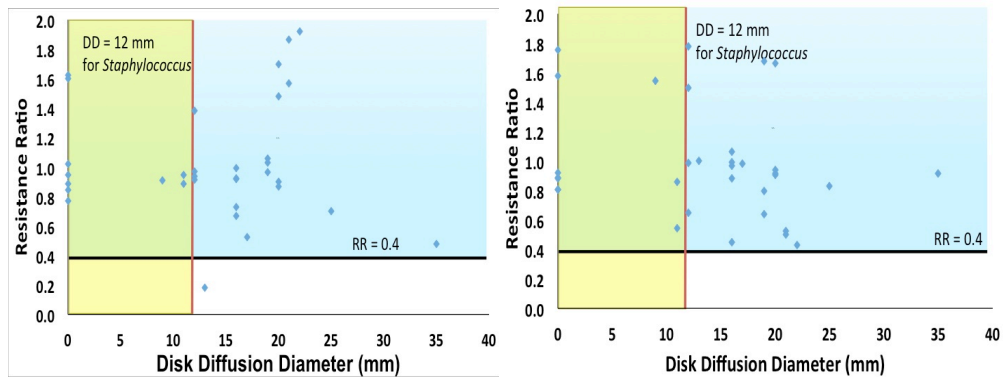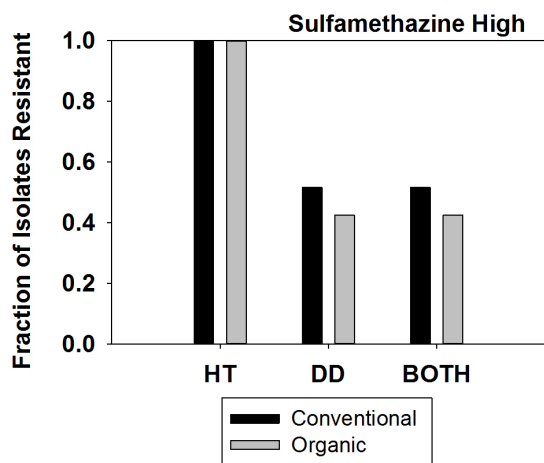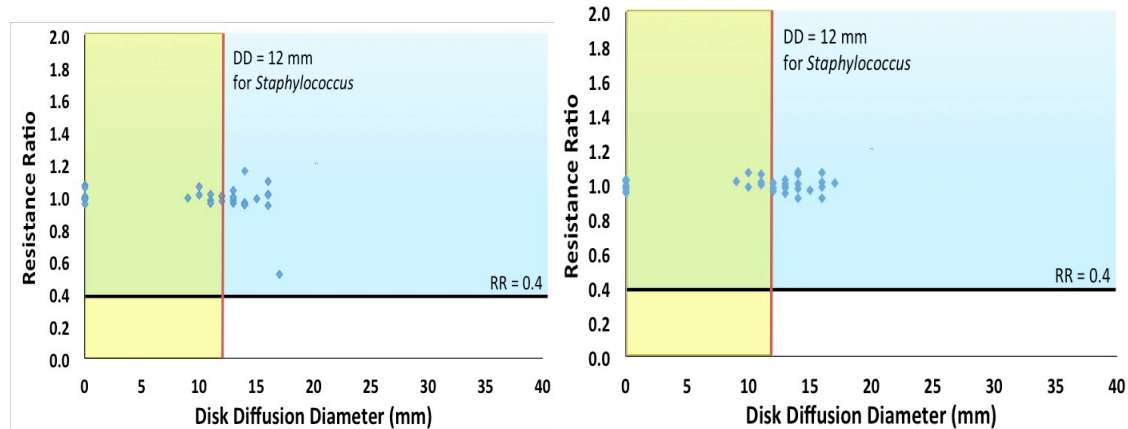**Figure 2 – 27.** Pattern 5: No difference among farm facilities. Oxytetracycline at high antibiotic concentration using PCA medium.
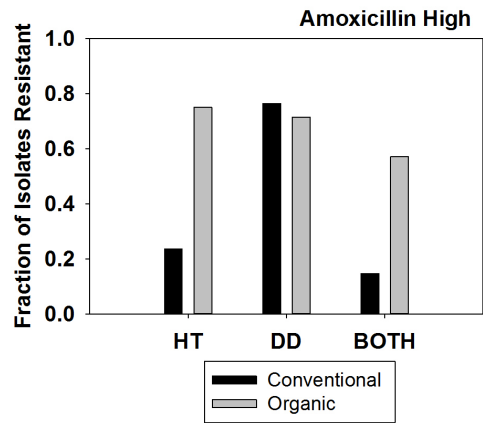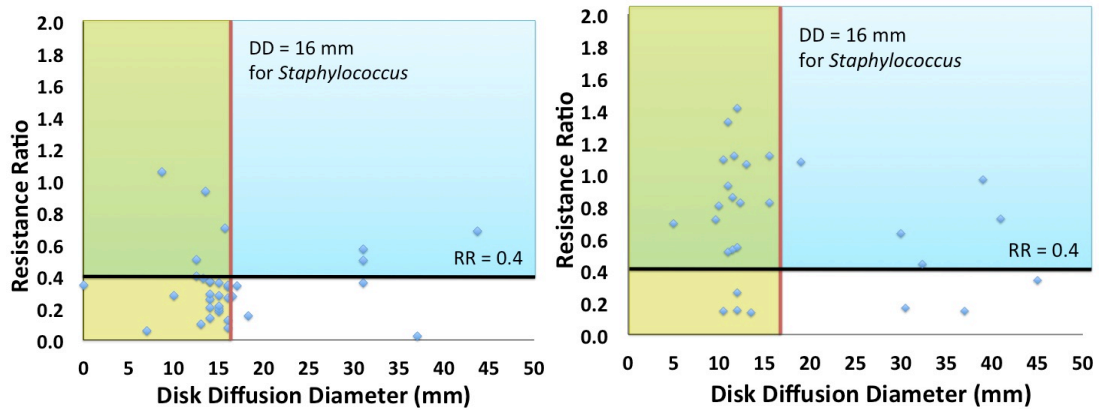
**Figure 2 – 28.** Pattern 6: Organic facilities show higher resistance than conventional facilities.

Amoxicillin at high antibiotic concentration using NA medium.

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# Chapter 3: Antibiotic resistance of *Escherichia coli* isolated from *Conventional, No Antibiotics,* and *Pristine Organic* chicken meat

**Abstract**

The use of antibiotics for therapeutic and non-therapeutic purposes in livestock farms promotes the development of antibiotic resistant bacteria. In this study, we cultured *Escherichia coli* from retail poultry falling into three categories of farming practices: *Conventional, No Antibiotics,* and *Pristine Organic*. We then examined the antibiotic resistance of the *E. coli* isolates (n = 424) by exposing them to seven common antibiotics via a high-throughput, liquid culture-based method: doxycycline, levofloxacin, cefoperazone, gentamicin, ampicillin, and oxytetracycline. For each isolate, resistance ratios (RR) were defined for each antibiotic as the change in $A_{600}$ over a certain time period in the presence of antibiotic over the change in $A_{600}$ for a control in the absence of antibiotic. Generally, the fraction resistant of the *E. coli* bacteria from the *Pristine Organic* was significantly lower than the *Conventional* and *No Antibiotic* categories, while the latter categories had similar fractions of isolates resistant. Specifically, for levofloxacin at a dosage of 4 mg/L, the *Pristine Organic* fraction of *E. coli* isolates resistant was a factor of approximately eleven and seven times smaller than the *No Antibiotics* and *Conventional* groups respectively. Similarly, for doxycycline at a dosage of 16 mg/L, the *Pristine Organic* fraction of *E. coli* isolates resistant was a factor of approximately two and two times smaller than the *No Antibiotics* and *Conventional* groups respectively. This study is the first of our knowledge to compare antibiotic resistance of *E. coli* from chicken sources including *Pristine Organic* brands.

## 1. Introduction

The World Health Organization (WHO) recently issued its first global report on antibiotic resistance, revealing a serious, worldwide threat to public health (1). While much attention has been focused on clinical overuse of antibiotics, up to 70% of antibiotics produced in the U.S. during 2008 were used for growth promotion in livestock alone (2). The practice of administering sub-therapeutic doses of antibiotics to prevent disease and promote growth in livestock can increase the resistance in bacteria found in both animals and humans (3-7). With regard to agricultural use, the WHO report states that "major gaps exist in surveillance and data sharing related to the emergence of ABR (antibacterial resistance) in foodborne bacteria and its potential impact on both animal and human health." Priority next steps include development of new tools and increased surveillance in food-producing animals and the food chain (1).

Both workers and consumers may be exposed to antibiotic resistant bacteria (ARB) as a result of farming practices. Poultry farm workers and families living on farms using antibiotics in the feed, as well as the neighboring families, had an elevated risk of exposure to antibiotic resistant *E. coli* (3,7,8). Consumers that purchase poultry products which utilize antibiotics in production may also be affected through consumption and cross-contamination on surfaces. To minimize their exposure, consumers should be adequately informed of antibiotic usage in retail poultry.

Labels on retail poultry can be a source of much confusion. Consumers are often presented with a wide variety of statements on labels regarding the quality of meat and levels of antibiotic usage during poultry production. Chickens raised with sub-therapeutic doses of antibiotics in conventional farming practice may still contain labels claiming "all natural" or "free range." Both of these statements are silent on antibiotics use[9]. For retail poultry meat, the

label "organic", as defined by the USDA, requires that animal are not given antibiotics or hormones after the first 24 hours of life. Thus, injecting antibiotics into eggs or administering antibiotics to chicks during the first day of life would not violate the USDA organic standard.

There are various forms of "No Antibiotic" labels in use, such as Raised Without Antibiotics (RWA), No Antibiotics Ever, No Antibiotics, etc. However, only RWA has a defined standard from the USDA (10). For the rest of the labels, producers develop their own antibiotics standards and terminology and present them for approval to the USDA FSIS (Food Safety and Inspection Service), but application materials are not made public. Additional certification allows for companies to provide an extra assurance to consumers that the process claimed was verified by either USDA or a third party (10,11).

For our purposes, we assign three different categories based on third party certification and statements on the retail labels: *Conventional, No Antibiotics* and *Pristine Organic*. Our *Conventional* products are assumed to be treated with antibiotics subtherapeutically. The *No Antibiotics* category includes brands which make reference to no antibiotic usage but may share facilities for slaughter with brands employing antibiotics. *Pristine Organic* poultry prohibits sub therapeutic use of antibiotics, ionophores, beta agonists, and/or sulfa drugs before and after hatching, provides vertically integrated production (no sharing of slaughter facilities, for example) and is third party certified. Vertical integration indicates that birds are raised from birth through slaughter by the same company without sharing of facilities. See Table 1 for information on the meanings of the categories with respect to antibiotic use and how brands were categorized.

*No Antibiotics, Conventional,* and *Pristine Organic* meat products provide various options to consumers, some of whom will pay a higher price for their preference according to

perceived health benefits (12). However, exposure to antibiotic resistance from these products cannot currently be discerned from labels. Some previous studies have shown higher antibiotic in resistance in conventional versus organic or "antibiotic free" poultry (13-15) while others have shown the converse or no difference (16-18). To our knowledge, there are no published data available on the frequency of ARB from *Pristine Organic* meat products.

This study compares the fraction of *E. coli* resistant to antibiotics among a variety of poultry products available in markets common in Southern California. We cultured *E. coli* isolates (n = 463) from wings, drumsticks, and chicken breast from several brands and used a high-throughput culture-based method for testing resistance to six antibiotics. We tested the hypothesis that there would be differences in antibiotic resistance among *Conventional*, *No Antibiotics* brands, and *Pristine Organic* brands. To our knowledge, this is the first study to test antibiotic resistant *E. coli* in *Pristine Organic* retail meat sources.

## 2. Materials and Methods

### 2.1. Meat Selection and Bacterial Purification

Raw chicken was purchased in shops in Los Angeles. Based on labels, the eight brands were grouped into the three categories of the study: *Pristine Organic* (PO-1, PO-2, PO-3), *Conventional* (C-1, C-2, C-3), and *No Antibiotics* (NA-1, NA-2). After purchase, products were stored at 4° C overnight until processing the next morning. Five samples of meat, each taken from a separate piece of meat, were tested for each type of meat (drumstick, wing, breast) from each brand. Each sample was aseptically removed from manufacturer packaging, and ~100 gram samples were weighed out. Samples were transferred to individual sterile Stomacher bags (VWR, Radon, PA, USA, catalog number 11216-902) and 125 ml of MacConkey Broth was added (18). The bag was then sealed and placed on a platform shaker (Barnstead Thermoclyne

Roto Mix, 50800) for five hours at 25°C at level 3 to allow sufficient dislodging of bacteria (most other studies have used a 24 hour contact time, but our goal was to minimize enrichment while allowing sufficient time for extracting bacteria). Immediately afterwards, 50 μl of the broth from each sample were spread onto three VRBA (violet red bile agar) plates, producing 15 VRBA plates for each meat brand (5 samples x 3 plates), as in Millman *et al.* (2013) (19). These plates were incubated at 35°C for 24 hours and 75 *E. coli* colonies were randomly chosen from the 15 VRBA plates for each meat brand and streaked onto CHROMagar plates (Hardy Diagnostics, Santa Maria, CA), for selection of *E. coli*. Then, each of the 75 *E. coli* isolates was purified twice on VRBA plates, and then lastly again on CHROMagar. Each purification plate was incubated at 37°C for 24 hours. The numbers of isolates that went through high throughput testing for antibiotic resistance are as follows:  41 C-2 Wings, 47 C-2 Drums, 40 C-1 Wings, 24 C-1 Drums, 39 NA-2 Drums, 19 NA-1 Wings, 44 C-3 Breast, 50 PO-1 Breast, 37 PO-2 Breast, 29 PO-2 Drums,  22 PO-3 Breast, 32 PO-3 Drums.

**Antibiotic Resistance Testing: High-throughput**

Antibiotic resistance testing using high –throughput (HT) was used to analyze each isolate for antibiotic resistance. A 1:40 dilution of isolate into Mueller-Hinton broth (a commonly used medium for antibiotic susceptibility testing, obtained from NCCLS 2002 guidelines (20) was used when dispensing antibiotics and isolates into high- throughput 384 well plates. 95 ul of Mueller-Hinton broth was pipetted into the 384 well plates onto rows A-P and columns 1-24. 2.5 ul of each antibiotic at high and low concentrations was administered. High and low antibiotic were as follows: doxycycline at 128 mg/L and 16 mg/L; levofloxacin at 32 mg/L and 4 mg/L; ampicillin at 512 mg/L and16 mg/L; cefoperazone at 512 mg/L and 128 mg/L; gentamicin at 64 mg/L and 8 mg/L; and oxytetracycline at 32 mg/L and 4 mg/L.

All six antibiotics chosen are relevant in both livestock agriculture and human medicine. These antibiotics also represent a range of distinct antibiotic classes: gentamicin is an aminoglycoside, levofloxacin is a quinolone, ampicillin is a penicillin, cefoperazone is a cephalosporin, and doxycycline and oxytetracycline are tetracyclines.

After the isolates were distributed into the high-throughput well plates, the bacteria growth was measured through absorbance at 600 nm (GloMax Multi Detection System). Absorbance readings were collected at the 0th, 4th, 24th, and 48th hour. Resistance ratios (RR) were defined for each isolate as the change in $A_{600}$ over a certain time period in the presence of antibiotic over the change in $A_{600}$ for a control in the absence of antibiotic. A high RR value (max of 1) indicated an isolate resistant to the antibiotic and a low RR value indicated an isolate more susceptible to the antibiotic. Two types of data analysis were used to compare RR among brands; one was by using the average growth among isolates by antibiotic by brand. Isolates were also analyzed by designating an RR cutoff value of 0.6 and determining the fraction of isolates resistant by antibiotic by brand. RR's considered as resistance ratio outliers (RR < -2 and RR > 2) were omitted from analysis.

**Antibiotic Resistance Testing: Disk Diffusion**

The standard method of measuring for antibiotic resistance, disk diffusion, was also used to test the same isolates (173 conventional, 42 organic, 166 pristine organic) in triplicate. In this method, each bacterial isolate (50 μL) was uniformly spread onto the surface of a Mueller-Hinton agar (MHA) plate with an antiseptic metal spreader to form an even film. Antibiotic paper disks for levofloxacin (5μg), doxycycline (30μg), oxytetracycline (30μg), gentamicin (10μg), cefoperazone (75μg), ampicillin (10 μg) (6 mm in diameter; BD Diagnostic Systems) were placed on the surface of each seeded MHA plate using a sterile pair of forceps. Plates were

incubated at 35°C for 16-18 hours. During incubation, antibiotic agents diffuse outwards, creating regions of inhibition within the microbial lawn. The diameters (mm) of the zones of inhibition were measured by a ruler or caliper. Based on the diameter of the inhibition zone and the CLSI interpretive criteria, the isolates are typically assigned to three categories: susceptible, intermediate, or resistant. For this study, the intermediate and susceptible categories were grouped. The smaller the diameter of the inhibition zone, the more resistant is the microorganism to the antibiotic.

## 3. Results

Isolates from conventional, organic, and pristine organic groups were both analyzed via HT method, which involved growing in liquid culture, and also analyzed via the DD method, which involved growing in a biofilm culture. Thus, resistance in both biofilm and planktonic growth modes could be assessed. Figures 1-7 shows scatterplots of the results from both methods. These graphs can be divided into four quadrants. Isolates characterized resistant by the HT and DD methods are on the top half, the left half and right half, of the plots, respectively. Orange horizontal line shows RR=0.6 which corresponds to DD for that antibiotic for *Escherichia coli.* Isolates characterized as resistant in both planktonic and biofilm growth modes fall in the top left quadrant. Notably, the upper right quadrant shows isolates that would be susceptible by DD but are resistant as characterized by HT. Data from the scatterplots are also presented as bar charts (Figures 1D-7D) comparing the fraction of resistant isolates from organic and conventional farms by HT, DD, and by both methods.

Several patterns emerged from the analysis by both methods. First pattern showed conventional and organic isolates show similar resistance for antibiotics: levofloxacin at low concentration, doxycycline at low and high concentration, oxytetracycline at high concentration,

cefoperazone at high concentration, gentamicin at high concentration, and ampicillin at high

concentration. For example, for doxycycline, gentamicin, oxytetracycline, cefoperazone, and

levofloxacin show organic either with similar resistance or greater than conventional which are

statistically significant (Figures 1D-7D) In the case of oxytetracycline, organic isolates have

greater isolates of fraction resistant than conventional isolates, 0.96 and 0.64, respectively. Only

one antibiotic showed a non statistical difference: ampicillin shows a significant difference

between conventional and organic for DD, but no statistical difference when using HT.

Second pattern showed pristine organic brands to have significantly lower resistance than

conventional and organic brands for 4 cases: levofloxacin, doxycycline, oxytetracycline,

gentamicin (Figures 1D-7D). Levofloxacin markedly showed a significant difference between

pristine organic (0.05, fraction of isolates resistant) and conventional (0.36, fraction of isolates

resistant) and organic brands (0.56, fraction of isolates resistant) (Figure 7D). Doxycycline (at

low concentration) as well showed a difference among pristine organic, conventional, and

organic with values: 0.47, 0.85, 1.0 (Figure 1D).  Third pattern showed planktonic method

showing a difference between conventional, organic, and pristine organic than biofilm method

(Figures 1ABC - 7ABC). Notably, many isolates fall in upper right that would be susceptible by

DD but are resistant as characterized by HT.  Doxycycline (at low concentration) has more

conventional isolates falling in the top right quadrant (Figure 1A) than the pristine organic

isolates (Figure 1C). This can be said for high concentrations of doxycycline, as well (Figure 2A,

Figure 2C). Levofloxacin also shows a marked difference in top right quadrant, where

conventional isolates fall under considerably more than pristine organic isolates (Figure 7A,

Figure 7C, respectively).

71

**4. Discussion**

In this work, *E. coli* isolates from *Pristine Organic* poultry were significantly less resistant to several antibiotics than those from either the *No Antibiotics* and *Conventional* categories of meat, suggesting that the fraction of antibiotic resistant *E. coli* differs depending on the type of poultry production system. In this study, the fraction of antibiotic resistant *E. coli* among conventional and organic isolates were either similar or organic isolates had a higher fraction of antibiotic resistant *E. coli* than conventional isolates. Another pattern found illustrated was pristine organic brands having significantly lower resistance than conventional and organic brands for 4 antibiotics: levofloxacin, doxycycline, oxytetracycline, and gentamicin. The third pattern found was how notably many isolates fell in the upper right quadrant of scatterplots for several antibiotics (doxycycline, levofloxacin, oxytetracycline, and gentamicin). This last finding was notable since it illustrated isolates susceptible by DD but are resistant as characterized by HT suggesting the need of the HT as an antibiotic resistance testing method.

Previous literature has had conflicting results with respect to antibiotic resistance comparisons in various types of meat products. Bacteria on meat from organic poultry farms were shown to have lower antibiotic resistance compared to conventional farms for *Campylobacter* (14, 21), *E. coli* (15, 22) and *Salmonella* (22) found that conventional meat brands had higher odds of carrying antibiotic resistance than antibiotic-free chicken products. Zhang *et al.* (2011) (22) found that *E. coli* and *Enterococcus spp*. on conventional retail meat are more likely to be more resistant to some antibiotics than on samples with labels stating "no antibiotics."

Conversely, other studies have found ARB resistance higher in organic products or having similar levels of ARB among meat products regardless of farming practice. Farming

practice showed similar frequency of antibiotic resistance when conventional, organic, and RWA

poultry brands were compared in a study conducted by Millman et al (2013) (19).  Liu *et al.*

(2012) (17) reports that there was more frequent occurrence of penicillin and ampicillin resistant

*Enterococcus* found in free range poultry than in conventional poultry in two Provinces of China.

Obeng et al. (2012) (18) found resistance to bacitracin, erythromycin, and tetracycline in most of

the isolates collected from both conventional and free range poultry and concluded that there was

no significant difference in antibiotic resistance in *Enterococci* between both types of poultry

farming.  Saleha *et al.* (2009) (23) found antibiotic-resistant *E. coli* in day old chicken on

commercial farms before introduction to any feed and water, therefore one possible explanation

for the similar resistance levels in conventional and organic poultry could be due to

contamination in the farming facilities.

In our study, levofloxacin showed to have the most striking difference between pristine

organic and conventional isolates. Levofloxacin is a new third generation fluoroquinolone

effective against bacteria such as Staphylococci, Streptococci, Enterobacteriaceae*, E. coli.*

Fluoroquinolones are a group of agents widely used both in human and veterinary medicine and

their antibiotic effect is through the inhibition of DNA gyrase, interfering with the supercoiling

of the bacterial chromosomal material. Levofloxacin is an antibiotic that is widely used in human

medicine, but veterinary medicine is seeking to adopt it for its usage. Levofloxacin is an

antibiotic that is widely used in human medicine, and may be adopted for veterinary medicine.

Studies are emerging where pharmacokinetics are being done on broiler chickens to understand

the appropriate withdrawal time of chickens after given dosages of levofloxacin (24, 25).

Kyuchukova et al 2013 suggests chicken producers to be aware of the withdrawal period of

levofloxacin used in their farms; they proposed an 8 day withdrawal period for all parts of chicken except the liver, where high levels persisted after the pharmacokinetic study.

Many studies reported that biofilms are more antibiotic resistant than planktonic cells (26,27,28). When comparing resistance between biofilm culture and planktonic cultures, there is typically a 10-1,000 fold difference in susceptibility. Biofilm culture are known to have a compact nature with extracellular polymetric surfaces separated by water channels ensuring the diffusion of oxygen, nutrients, and waste product. Biofilms have a level of protection due to their physiology along with their metabolic and oxygen gradients; these gradients reduce the antibiotics effect that target growth and metabolic processes. (29) While it is generally thought that biofilms having greater resistance than planktonic cells, one study claimed that this premise was not justified since biofilm cultures depend largely on persister cells (30). Their study suggests that persisters, cells that persist living but with a disabled PCD mechanism, is what allows for planktonic cells to have the advantage in antibiotic resistance over biofilm cells. Other studies concluded that persister accumulation can occur if mutations happen increasing the number of persisters in exponential phase in *E. coli* (31,32,33).

Resch et al 2004 (34) demonstrated through side by side planktonic and biofilm testing that the gene expression patterns are distinct under the two growth conditions. Biofilm cells had their cell envelope as the most active compartment: cell wall synthesis and function were up-regulated. Conversely, planktonic cells had toxins and proteases up-regulated.

This work and previous literature show that retail chicken meat cannot easily be categorized due to various factors including sharing of slaughterhouse facilities and possible antibiotic use during the first 24 hours of life, which is allowable under the designation of "Organic." While limiting in scope, our findings suggest that *No Antibiotic* poultry, unless

74

*Pristine Organic*, may be just as likely to harbor ARB as *Conventional* product meats. The results suggest that more stringent and detailed federal regulation over labels is needed. Additional work should focus on the prevalence of antibiotic resistant bacteria among meat raised with various practices.

**Tables**
**Table 3 - 1:** Categories of retail poultry meat, labels, and definitions for this study.

| Category | Brand Name Designation | Labels | USDA label approved? | Third Party Certification | Other Labels |
|---|---|---|---|---|---|
| *No Antibiotics* | NA-1 | No Antibiotics Ever | Yes (company's definition approved) | No | All Natural: Minimal processing, no artificial ingredients, all vegetarian fed, no added hormones |
| | NA-2 | Organic | Yes | Yes after 24 hours of birth (Organic seal) | Organic: Animals are raised in conditions that accommodate their behavior and fed organic feed, not given antibiotics or hormones after 24 hrs. of life |
| | | RWA | Yes | No approval for RWA | Free Range: Access for all animals to outdoors<br><br>Raised without antibiotics,: |
| *Conventional* | C-1 | All Natural | Yes | No | All Natural: Minimal processing, no artificial ingredients, all vegetarian fed, no added hormones |
| | C-2 | All Natural | Yes | No | |
| | C-3 | All Natural | Yes | No | Minimal processing, no artificial ingredients, has no statement of use of antibiotics |
| *Pristine Organic* | PO-1 | Organic | Yes | Yes (Global Animal Partnership) | Free Range: Access for all animals to outdoors<br><br>Air Chilled<br><br>Vegetarian Diet, No Preservatives, Raised without added hormones Vertically integrated production that uses all common organic standards |
| | PO-2 PO-3 | No Antibiotics Ever | Yes | Yes (Global Animal Partnership) | |

**Figures**

**A**



**B**



**C**



**D**



**Figure 3 - 1.** Doxycycline at a low concentration. A) Conventional B) No Antibiotics C) Pristine

Organic D) Both methods showing fraction of isolates resistant per group: conventional, organic,

pristine

**Figure 3 - 2.** Doxycycline at a high concentration. A) Conventional B) No Antibiotics C)

Pristine Organic D) Both methods showing fraction of isolates resistant per group: conventional,
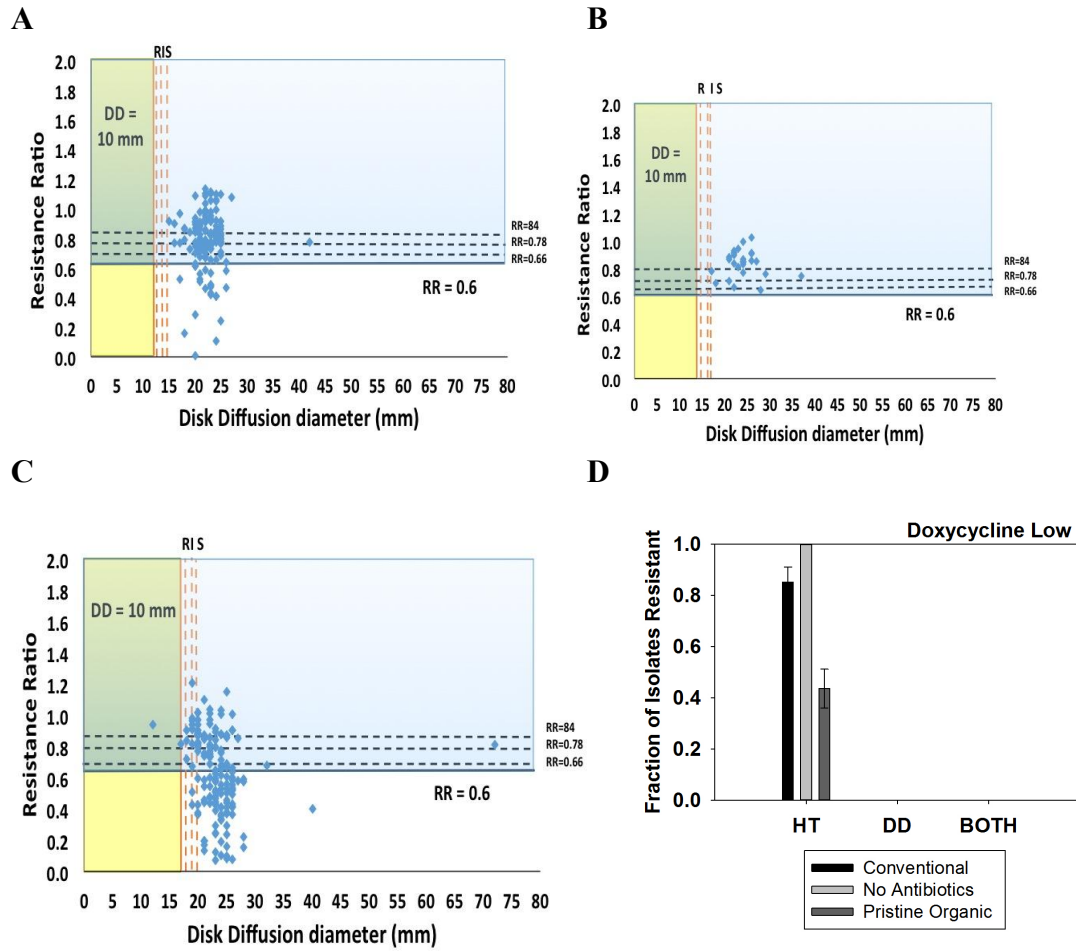
organic, pristine

**Figure 3 - 3.** Oxytetracycline at a high concentration. A) Conventional B) No Antibiotics C) Pristine Organic D) Both methods showing fraction of isolates resistant per group: conventional, organic, pristine
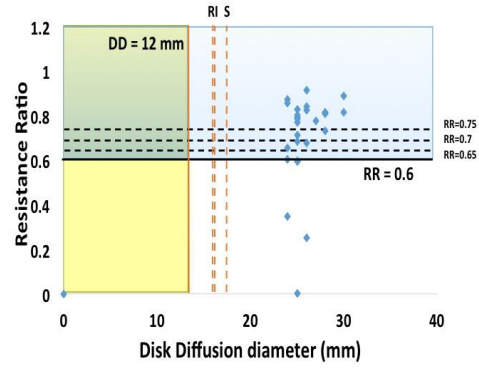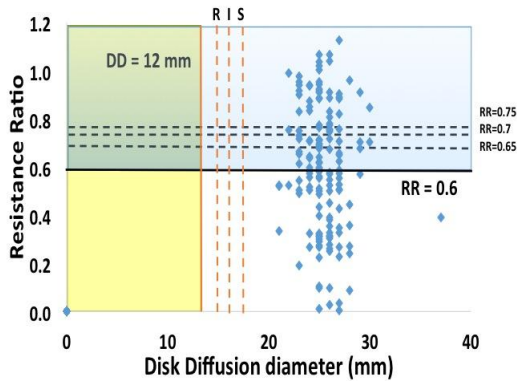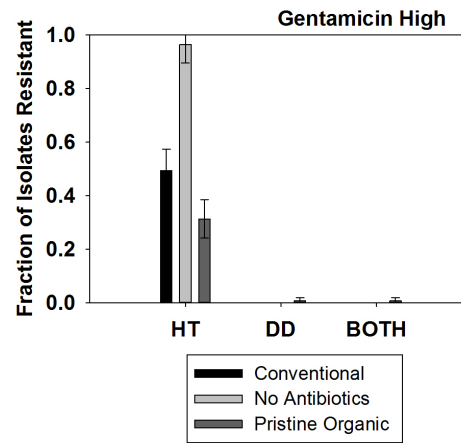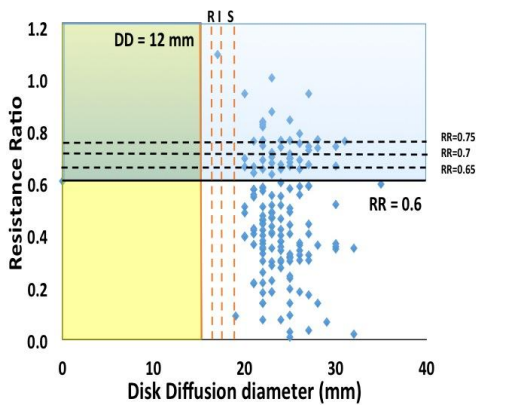
**Figure 3 -4.** Gentamicin at a high concentration. A) Conventional B) No Antibiotics C) Pristine Organic D) Both methods showing fraction of isolates resistant per group: conventional, organic, pristine
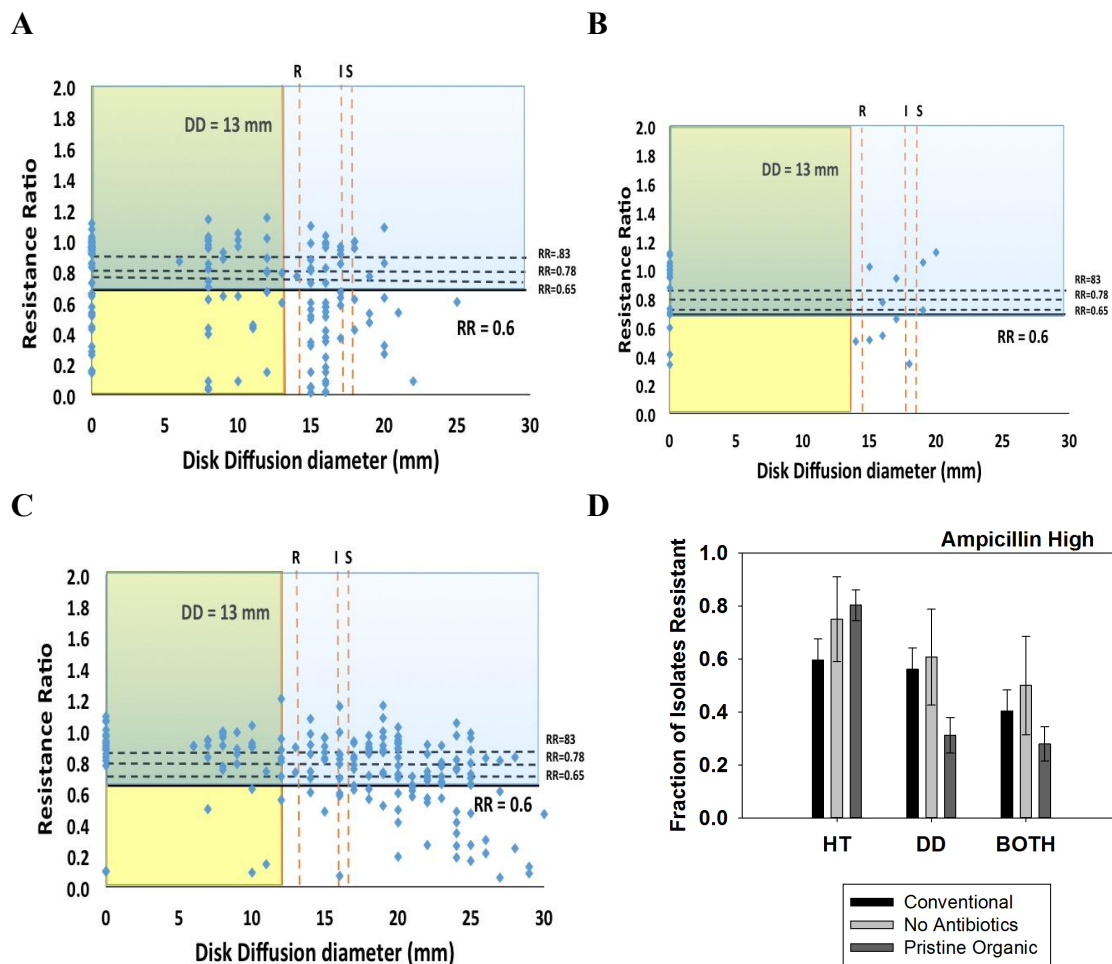
**Figure 3 -5.** Ampicillin at a high concentration. A) Conventional B) No Antibiotics C) Pristine Organic D) Both methods showing fraction of isolates resistant per group: conventional, organic, pristine
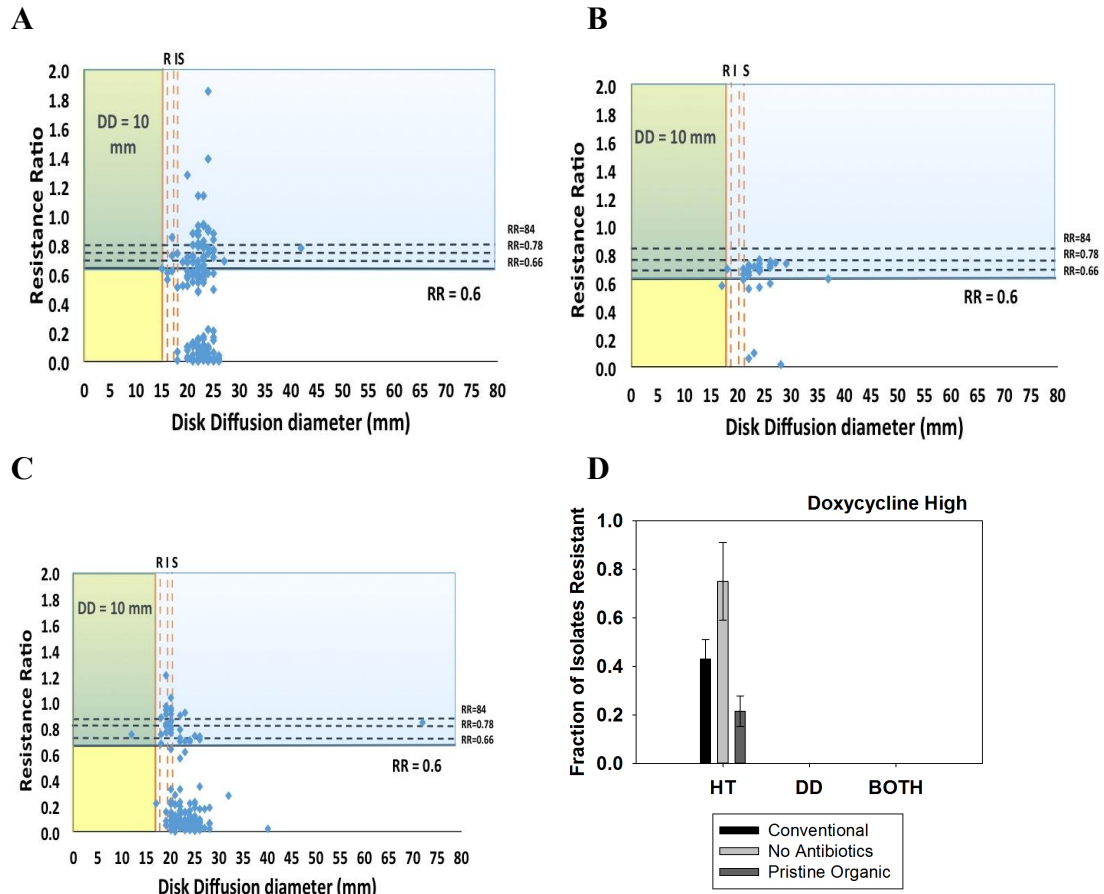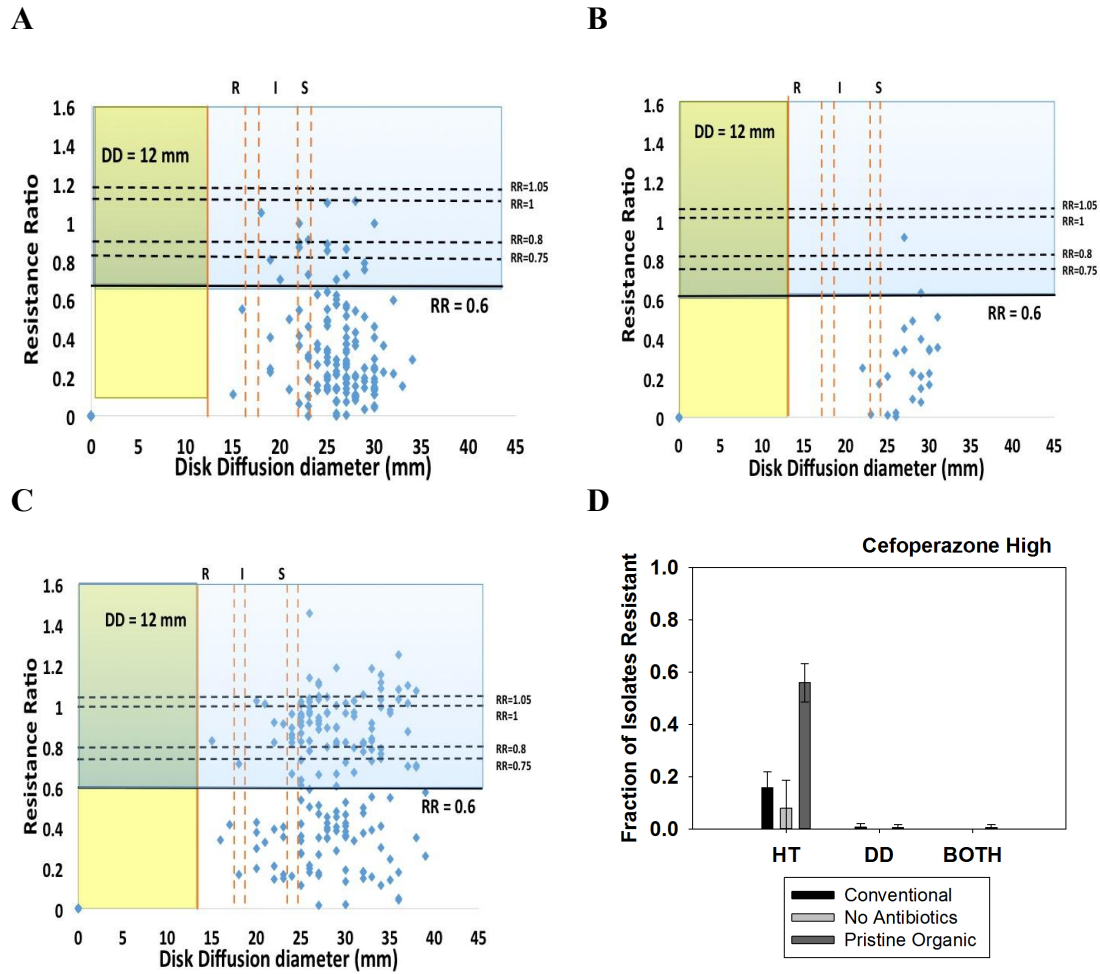
**Figure 3 -6.** Cefoperazone at a high concentration. A) Conventional B) No Antibiotics C) Pristine Organic D) Both methods showing fraction of isolates resistant per group: conventional, organic, pristine
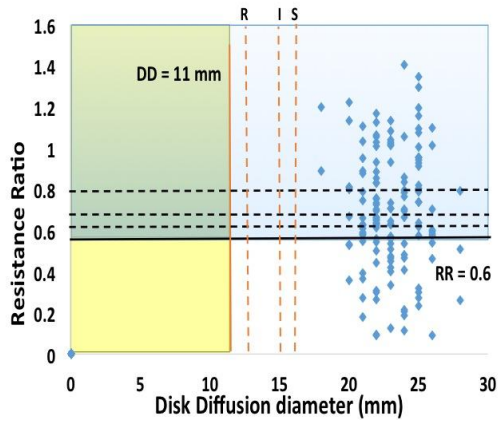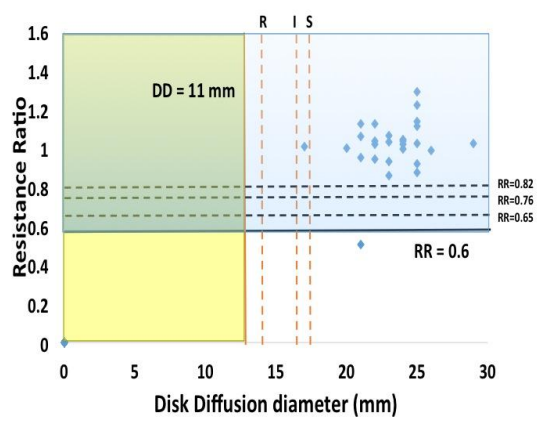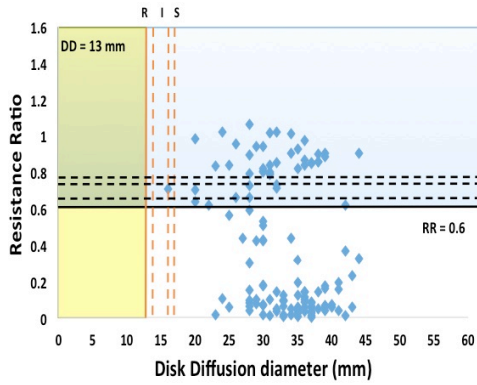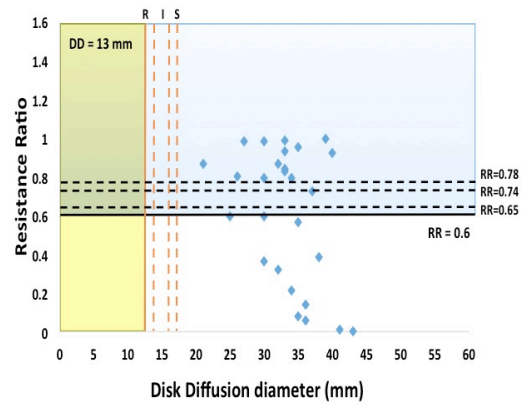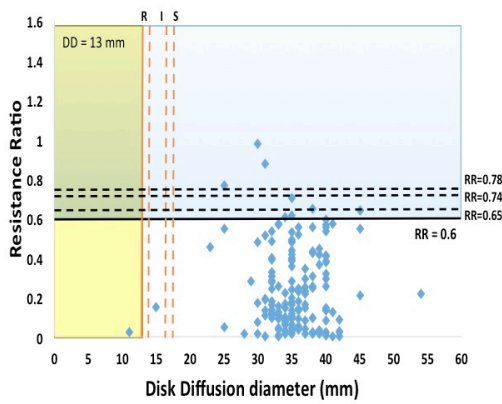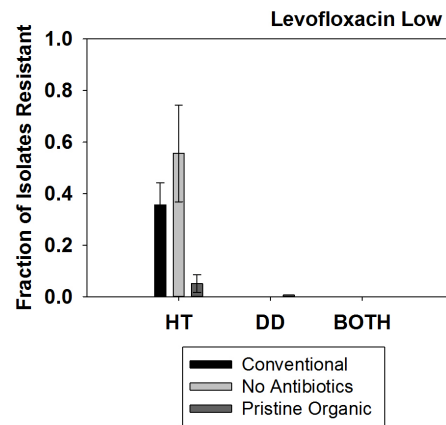
**Figure 3 -7.** Levofloxacin at a low concentration. A) Conventional B) No Antibiotics C) Pristine

Organic D) Both methods showing fraction of isolates resistant per group: conventional, organic,
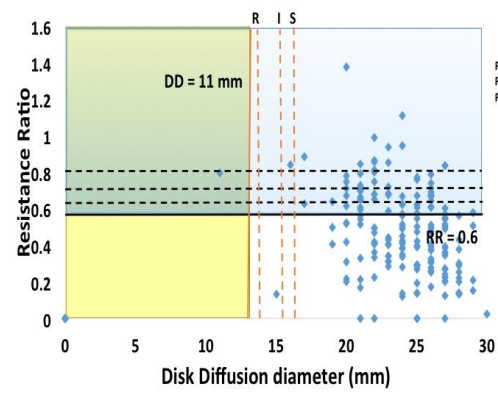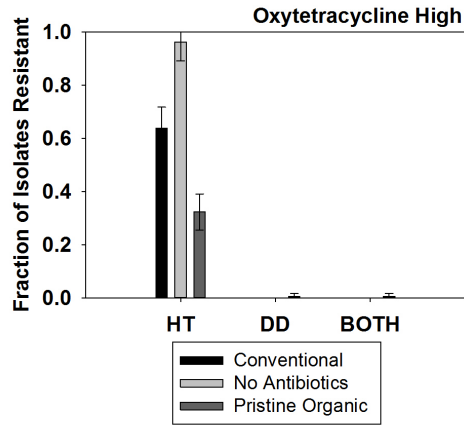
and pristine
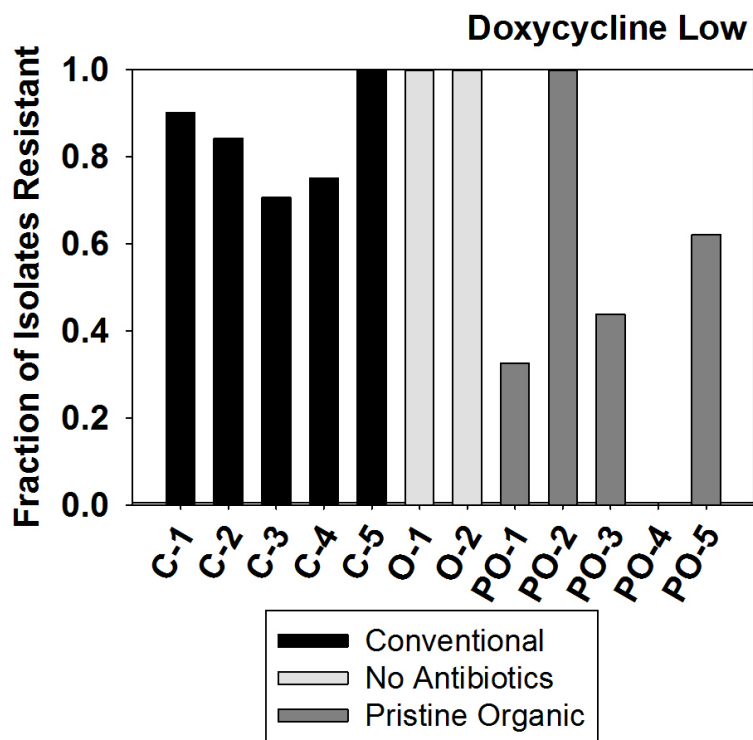
**Figure 3 - 8.** Doxycycline at a low concentration.
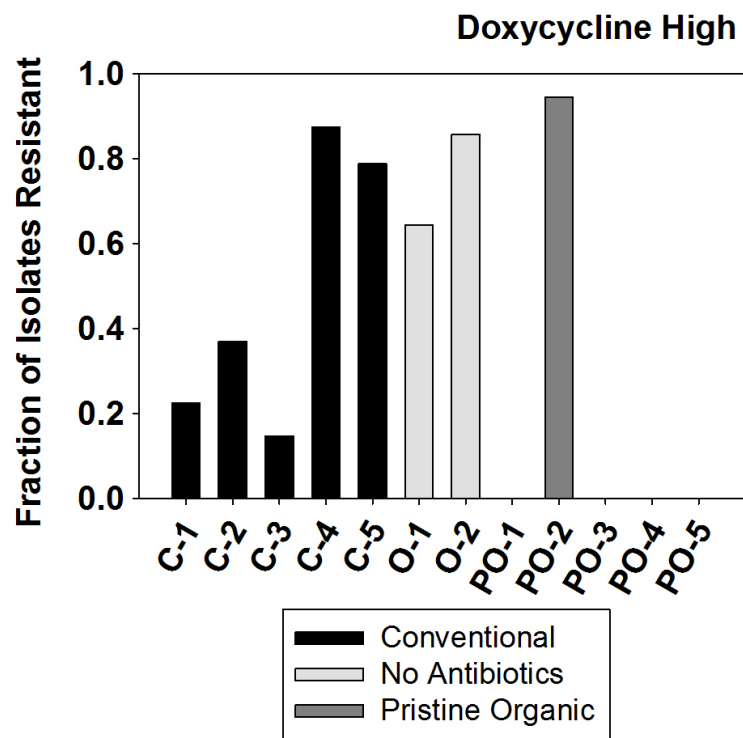
**Figure 3 - 9.** Doxycycline at a high concentration.
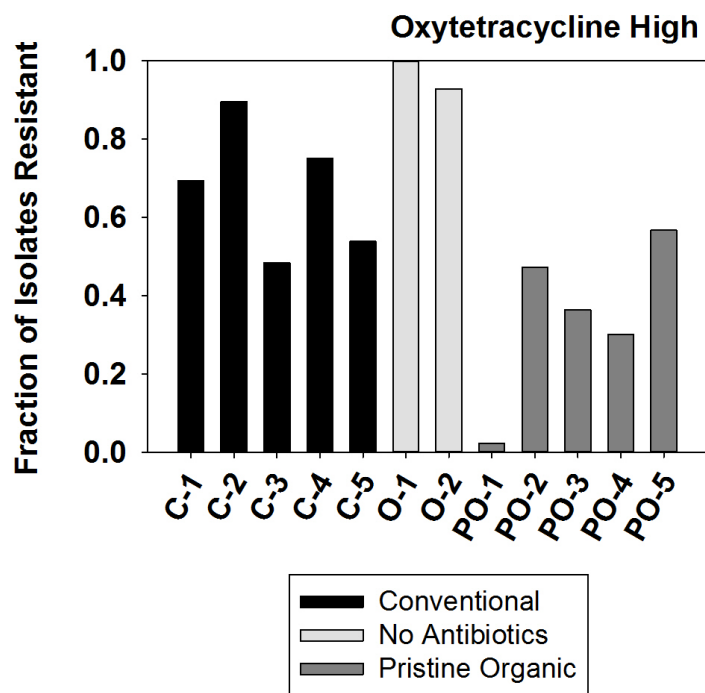
**Figure 3 - 10.** Oxytetracycline at a high concentration. .
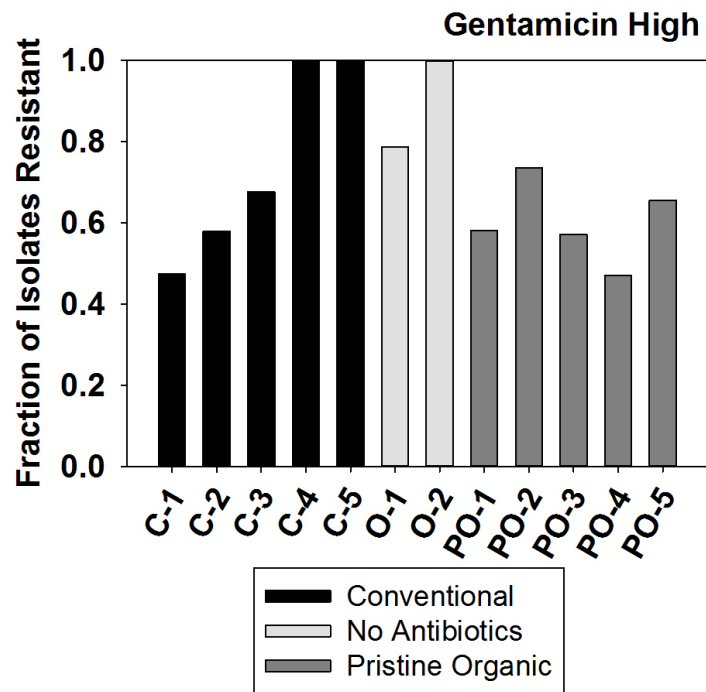
**Figure 3 - 11.** Gentamicin at a high concentration.
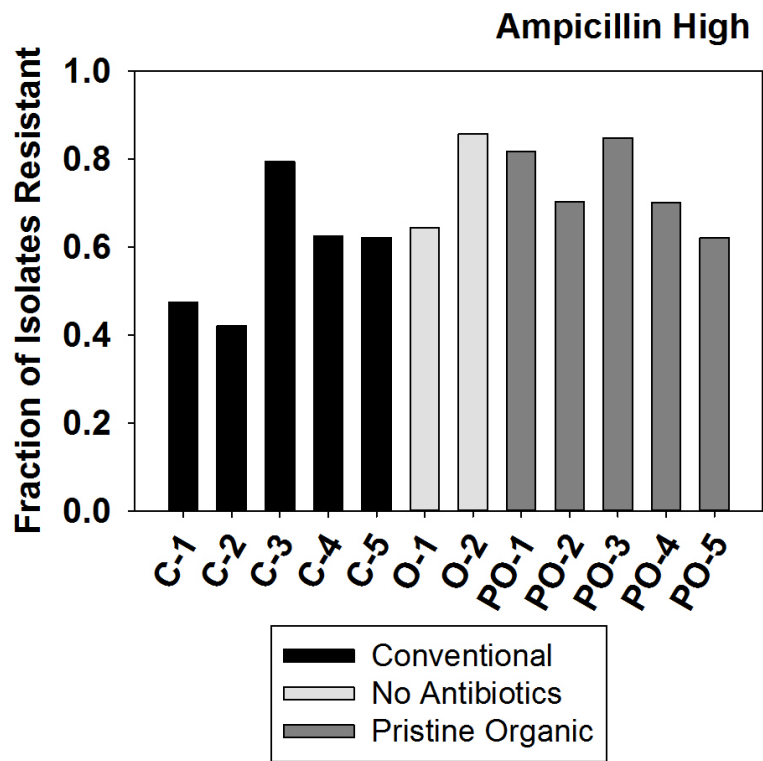
**Figure 3 - 12.** Ampicillin at a high concentration.
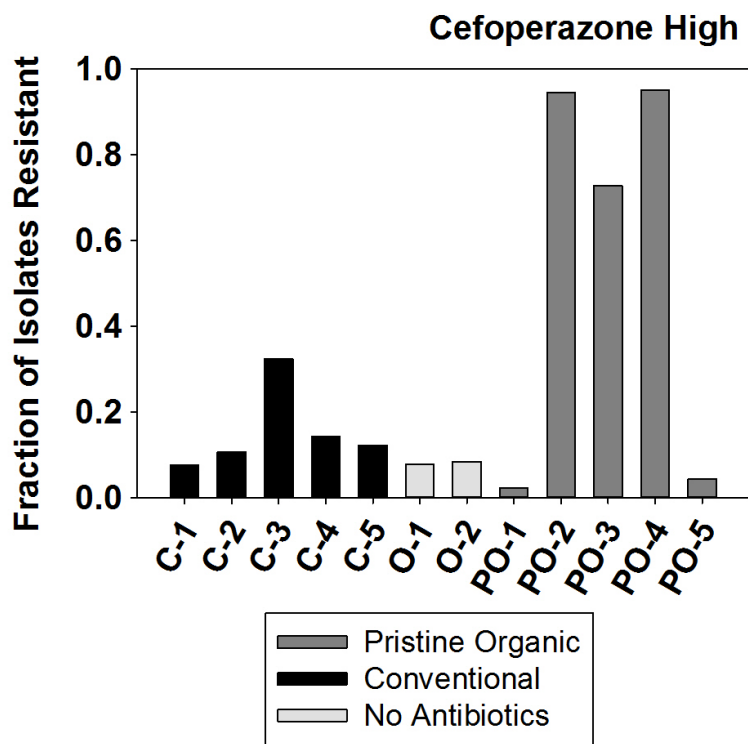
**Figure 3 - 13.** Cefoperazone at a high concentration. .
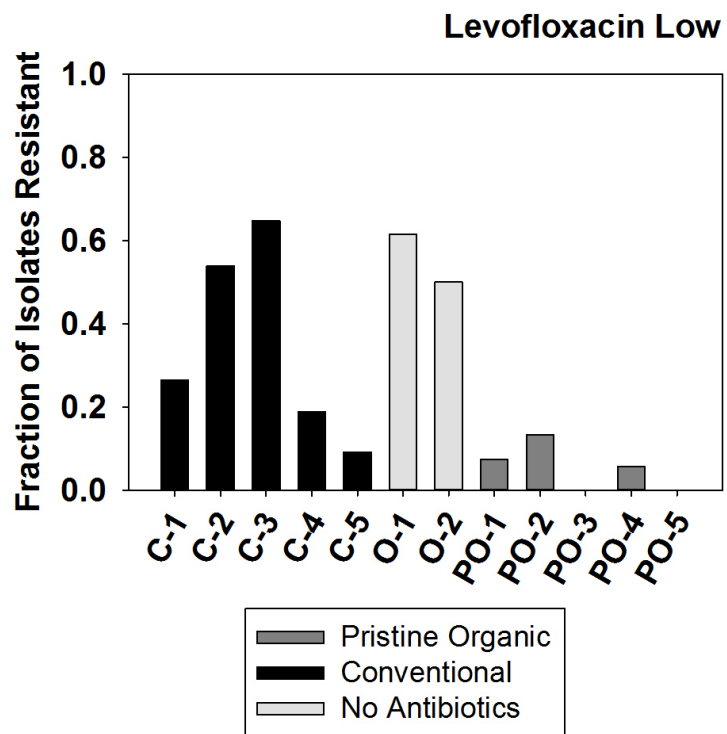
**Figure 3 - 14.** Levofloxacin at a low concentration. .

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**Chapter 3: Airborne Antibiotic Resistance Genes Upwind and Downwind of CAFO Poultry Sites**

**Abstract**

The development of antibiotic resistance in bacteria as a result of such widespread antibiotic use has been recognized as a threat to society since the 1960's. CAFOs (confined animal feeding operations) can create environments for bacterial populations that inevitably propagate antibiotic resistance within animals and humans. This can allow for the antibiotic resistant genes (ARGs) to spread into the surrounding environment. Most studies focus on presence of ARGs via water and soil pathways from agricultural settings. However, similar impacts through air are scant. Since airborne antibiotic resistant genes from poultry confined animal feeding operations (CAFO's) is limited, our study aimed to investigate for antibiotic resistant genes of bioaerosols from upwind and downwind locations of poultry CAFOs in the Central Valley, Califronia. This area holds extensive agricultural activity. We sampled fours poultry CAFO sites in the Central Valley and also a control site. For all cases where ARGs were observed, downwind samples showed a higher level of ARG's for $bla_{shv}$ and $erm$(F) than upwind samples both were absent at the control site and one of the agricultural sites. For example, one site showed the downwind prevalence of $bla_{SHV}$ was over 8000 times higher in concentration than that for the upwind sample. For another site, $erm$(F) prevalence downwind was 20 times higher in concentration that of upwind. Our study shows that the genetic material of antibiotic resistant bacteria can still persist downwind of the CAFO site. Our study suggests that poultry CAFOs can be a point source of ARG environmental pollution Regular surveillance of ARG from poultry sources is suggested to detect development of airborne antimicrobial resistance that can be spread to surrounding community, especially humans living near farms, via wind air

trajectories. The microbiome of family workers can be impacted by these ARG's and research into the public health should be investigated. To our knowledge, this is the first study to examine air samples collected upwind and downwind of poultry CAFOs for elevated levels of ARGs.

**Introduction**

Today, the use in antibiotics in livestock production greatly exceeds that of human applications. Approximately 24.6 million pounds of antibiotics are used annually in animal agriculture, compared to only 3 million pounds for human medicine (Mellon *et al*. 2001). The excessive use of sub-therapeutic applications of antibiotics in livestock production fosters the development of antibiotic resistant bacteria (ARB). The development of antibiotic resistance in bacteria as a result of such widespread antibiotic use has been recognized as a threat to society since the 1960's (Bulling 1973, Levy *et al*. 1976, Isaacson and Torrence, 2002, World Health Organization 2003, Institute of Medicine 2003, Silbergeld *et al*. 2008, World Health Organization 2014). In many countries around the world, antibiotic use in livestock production goes beyond the functional purpose of treating of sick animals and is instead used as a way to promote growth and prevent disease. Specifically, the invention of the U.S. broiler chicken industry transformed livestock production from independent farms to an industrial production (Martinez 2002, Graham 2008, Leibler 2009). While these new methods were instrumental in allowing farmers to meet food demands, the resulting increase in resistance quickly became apparent. Observed increases in ARGs have coincided with the addition of antibiotics into livestock operations (Endtz et al., 1991; Bager et al., 1997; and Nelson et al., 2007) and studies comparing ARG prevalence after bans of antibiotics use for growth promotions have observed decreases in resistance. (Aarestrup et al., 2001; Bager et al.. 1999; Bogaard, Bruinsma, and Stobberingh. 2000; Klare et al.. 1999; Pantosti et al., 1999; and Wegener et al., 1999). Amid

concerns of increasing antibiotic resistance, the European Union banned the use of antibiotics for growth promotion in 2006. The U.S. has yet to implement such bans but does suggest voluntarily reductions in use of antibiotics.

Confined animal feeding operations (CAFOs) create environments for bacterial populations that inevitably propagate antibiotic resistance within animals and humans (Davis et all 2011). This in turn changes the resistome (collection of ARGs in microbial communities) and allows the antibiotic resistant genes (ARGs) to spread into the surrounding environment (Wright 2007, Martinez 2009, Wright 2010). Several studies have shown how CAFOs can disseminate ARB into the environment through environmental pathways of air, soil, surface water, groundwater, as well as through the retail process for meat distribution (Gibbs *et al*. 2006; Green *et al*. 2012; Pruden *et al*. 2012). Sources of ARB dispersal have been identified and include the use of manure for land application (Heuer *et al*. 2011), air inside and downwind of CAFOs (Chapin *et al*. 2005, Gibbs *et al*. 2006), groundwater adjacent to such facilities (Sapkota *et al*. 2007), and air behind vehicles transporting animals (Rule *et al*. 2008).

Bacteria from fecal matter dust inside CAFOs have potential to become airborne and transferred to the outside environment via the large fans used to keep coupes highly ventilated (Price 2008). This contamination of surrounding air downwind of facility has been observed in swine CAFOs (Gibbs *et al*. 2006 and Green *et al*. 2006). The study found the presence of ARB inside the facilities and up to 150 meters downwind of a facility (Gibbs 2006). Additional studies have concluded that airborne methicillin-resistant *Staphylococcus aureus* (MRSA) could be measured inside a swine CAFO and 215 m downwind of the operation (Ferguson 2012). However, fewer studies have shown the release of ARB specifically from poultry CAFOs. ARB from poultry CAFOs present a risk because they can become airborne, contaminating external

98

local air nearby. Brooks 2010 found the prevalence of ARB in soil near poultry CAFOs, with higher levels correlated with proximity to the farm.

Growing evidence exists that ARGs are found via water and soil pathways similar impacts through air are non-existent. Dutch scientists started monitoring ARGs in agricultural soils as early as the 1940s and have observed an increase in antibiotic resistance gene abundances in soil over time, such as a 15-fold increase of ARGs today compared to the 1970s. The highest rates of increase were shown by tetracyclines (*tet*(Q), *tet*(O), *tet*(M) and the beta-lactamase, *bla*$_{TEM}$ (Knapp 2010). Common antibiotics used in agriculture include beta-lactamases and macrolides such as erythromycin. The resistance genes associated with these antibiotics have been studied to understand their fate in the soil environment. In several studies, *van*(A), *van*(B), *tet*(B), *tet*(L), *tet*(O), *erm*(B), *erm*(F), *bla*$_{TEM}$, *bla*$_{SHV}$ were chosen due to crossed drug classes and prevalence in environmental samples from agricultural origins (Knapp et al 2010, Peak 2007, Tzavaras *et al*. 2012). In Knapp et al. 2010, of eleven ARGs tested in samples from intensive agricultural operations, *erm*(B), *erm*(F), *bla*$_{TEM}$, *bla*$_{SHV}$ were found in abundance in the environment. That finding showed the importance of ARG release to the environment and called for best management strategies and environmental policies related to the handling of agricultural outlets to the environment (Knapp et al 2010). In the Peak (2007) study, ARGs (*tet*(O)), *tet*(Q), *tet*(W), *tet*(M), *tet*(B), *tet* (L), were tested at eight lagoons from CAFOs to understand how feedlot operations affect ARGs in downstream surface waters. This study found that *tet* gene levels were higher in abundance in lagoons that higher usage from farms than lagoons that had no usage.

Since airborne ARG's from poultry CAFO's is scant, our study aimed to examine for antibiotic resistant genes of bioaerosols from upwind and downwind locations of poultry CAFOs

To our knowledge, this is the first study to examine air samples collected upwind and downwind of CAFOs for elevated levels of ARGs.

**Materials and Methods**

*Study Site and Sample Collection*

In California, the Central Valley is an area of extensive agricultural activity. Therefore, the prevalence of animal farming in this area may alter environmental antibiotic resistance in surrounding airborne bacteria. Wind patterns are an important consideration as there is potential for differences in observed ARG concentrations upwind and downwind from the poultry farms. At each sampling site, temperature and wind direction were recorded (Table 1). Unstable atmospheric conditions results in increased respirable sized particles being locally dispersed. To confirm the orientation of upwind and downwind trajectories for each day and hour of sampling, the National Oceanic and Atmospheric Administration (NOAA) HYSPLIT trajectory model and database was used (Draxler et al. 2014).

Bioaerosols were collected near each conventional poultry farm site. The distances from sampling location to CAFO is provided for each site (Table 1). The CAFOs tested for were surrounded by agricultural activities that did not involve animal production. Air samples were collected using a Millipore glass fiber filters and pumps (Fisher Scientific, catalog number APFA 041 00). Samples were taken at downwind and upwind locations surrounding the farm. After collection, the samples were sealed in sterilized petri dishes and stored on ice for 8 hours. Then transferred to -80°C refrigeration until DNA extraction.

*DNA Extraction*

DNA was extracted using the PowerFecal DNA Isolation Kit (MoBio Laboratories, Inc, Carlsbad, CA, Catalog No. 12830-50) (Knapp, 2010, Knapp et al, 2010, Peak et al 2007). The filters were aseptically added to the extraction beads and buffer, and agitated for 2 min. 100 ul of Bead Solution was added to the extraction bead tubes to allow for more supernatant extracted in the initial steps. The samples were further purified following the manufacturer protocol. 100ul of DNA eluent were stored at -20°C for further processing.

*qPCR Methods*

The abundance of 9 ARGs were measured using real time qPCR (Applied Biosystems) (Table 4). *erm*(B), *erm*(F), *bla*$_{TEM}$, *bla*$_{SHV}$, *van*(A), *van*(B), *tet*(B), *tet*(L), *tet*(O) were chosen due to crossed drug classes and prevalence in environmental samples (Knapp, 2010, Knapp et al, 2010, Peak et al 2007). Table 4 provides the primers and reaction conditions used in the study. The ARGs of interest required both TaqMan and SYBR-green Master Mix kits to target genes. SYBR-Green requires a well volume of 25 μL; containing 12.5 μL of the 1X SYBR® Green Master Mix (Applied Biosystems), 2μL of the DNA samples, 1.25 μL of both forward and reverse primers, and 8 μL molecular grade H$_2$O. Taqman Master Mix (Applied Biosystems) also required a mixture reaction volume of 25 μL; containing 12.5 μL of 1X SYBR® Green Master Mix (Applied Biosystems), 2μL of the DNA samples, 1.25 μL of both forward and reverse primers, 1 μL of Taqman probe, and 7 μL molecular grade H$_2$O. Reactions are then performed in triplicate using ABI 7700 Sequence Detection System. Temperature cycle is 95°C (10min), and 45 cycles of 94°C (20s), and annealing and elongation conditions (Table 4), and melting curve of 50-95 °C, ΔT = 0.1 °C/second). Table 2 lists the primers and reaction conditions used in the study. Melting curve analysis was conducted to indicate correct positive amplification of target gene. Samples from each farm site were analyzed in triplicate and the threshold cycle (Ct) value was averaged for each sample. A positive detection for each sample was defined as at least two of three

replicate wells showing amplification with a Ct value less than 40. Positive control sequences for each gene were obtained from gBLOCK (IDT Technologies). The Ct values were converted into units of gene copies using a pooled master standard calibration model (a five-point standard calibration curve was taken similarly as ARG assays as described above) for curves having efficiencies between 90 – 110% and $R^2 > 0.99$.

**Results and Discussion**

Antibiotic resistance genes of $bla_{SHV}$ and $erm$(F) were analyzed for copy genes downwind and upwind from Sites 1 - 4, and the Control Site. Generally, downwind samples at most sites showed a higher frequency of ARG's for $bla_{shv}$ and $erm$(F) than upwind samples (Figure 2). For Site 1 the downwind location, $erm$(F) and $bla_{SHV}$ were 17 and 3 times higher in concentration (copies/$\mu$l) than the upwind site. For Site 2, $erm$(F) prevalence downwind was 20 times higher in concentration that of upwind. Site 3 produced no hits on ARGs, and for Site 4 the downwind prevalence of $bla_{SHV}$ was over 8000 times higher in concentration than that of upwind levels. None of the ARGs tested were present in the Control Site samples. The other genes analyzed: $erm$(B), $bla_{TEM}$, $van$(A), $van$(B), $tet$(B), $tet$(L), $tet$(O), were tested but no signal was found in any site (downwind nor upwind).

For sites at which ARG presence was found (sites 1, 2 and 4), downwind sampling locations ranged from 69 m to 127 m from the CAFO sites (measured from the edge of the nearest CAFO building, Table 1). The downwind sampling point for site 3 was located 445 m from the CAFO; no antibiotic resistance genes were found at either the downwind or upwind locations for this site. Dust dispersion under daytime conditions with moderate winds has been investigated around roadways; typical daytime dispersion distances from this dust source are under about 300m (Karner et al. 2010), thus the much larger distance for the downwind sampling

location for site 3 may explain the absence of ARGs at that site. Some upwind samples did show a small quantity of ARGs, such as Site 1 with approximately 500 copies/$\mu$l of $bla_{shv}$; whereas, downwind samples from the same site showed 4 times fold number of copies (approximately 2000 copies/ $\mu$l). While the reason for this is not certain, the most likely explanation is that these ARGs result from resuspended material from closer to the upwind site. Because the CAFOs were all at least 3600 m from the nearest neighboring CAFO, the ARGs may have persisted in the soil from when the wind previously traveled from the CAFO the upwind site (and then have been resuspended). The alternative mechanism; transport in the upwind direction would not be expected (over several hundred meters) under the atmospheric conditions present during sampling. While nearest weather station data the previous day or two did report a wind reversal during daytime, there were many times at night and during the early morning at all sites during which wind direction was not recorded; this is the standard reporting practice for conditions when winds are below 1 m/s or are variable. Under such low winds, stable/neutral atmosphere conditions, plume travel can 1000 m or more (Choi et al. 2012), thus there is the possibility that ARGs were delivered to the vicinity of the upwind site in the prior few nights.

There is little known about the viability of bacteria and the stability of ARGs in the environment. Marthi et al. (1990) investigated the survival of antibiotic resistant bacteria through aerosolization experiments. Their findings showed that the viability of bacteria decreased over 15 m (Marthi et al 1990). Other studies of non-resistant bacteria such as Hambelton et al 1983, which investigated the survival of *Legionella pneumophilia* and Tang et al 2009, which summarized that relative humidity and temperature were two common factors in survival of airborne bacteria, however indicate some bacteria have much longer survival times while airborne. This is important to note since even though the ARB may become less viable via

distance, its genetic material still persists such as shown in this study. Given the uncertainty about both bacteria and ARG stability in the environment, it is hard to know how recently a transport event would be needed to able to explain the observation of ARGs at the upwind sites.

Results show that ARGs are more abundance downwind of a poultry site than upwind in three of the four sites. Our study suggests that poultry CAFOs can be a point source of ARG environmental pollution. Livestock given antibiotics serve as breeding grounds for the emergence of new resistant strains of bacteria. In the gut bacteria of the animal, ARG operons can be accumulated on integrons and moved to plasmids and other moveable elements. AAU (agricultural use) selects for assembly of resistance gene clusters that then move to commensals and pathogens in the microbial community by horizontal gene transfer (HGT). HGT occurs between strains of the same species and between species via several mechanisms including plasmid transfer, phage transduction, and transformation. The result is a larger reservoir of ARB than occurs in the absence of selective pressure by antibiotics, and these ARB serve as vectors that move ARG to human microflora.

In contrast, ARG can also be transferred to commensal and pathogenic bacteria that are capable of human to human transmission. A recent study showed evidence for transmission of ARGs from environmental bacteria to pathogens.[48] Some bacteria, such as vancomycin-resistant enterococci can serve as pathogens in both animals and humans, and once they cross the species barriers can result in sustained illness in humans.[49] However, even non-pathogenic, commensal bacteria originating in animals are a source of ARG in the human microbiome that may subsequently be passed among species.

The higher frequency of ARG detection near downwind versus upwind of poultry farms collectively show the occurrence of antibiotic resistance in the environment, which may imply

104

potential exposure to ARGs via an air pathway downwind of poultry farm. Regular monitoring and surveillance of ARG from poultry sources is suggested to detect development of airborne antimicrobial resistance that can be spread to surrounding community, especially humans living near farms and workers, via wind air trajectories. The microbiome of family workers can be impacted by these ARG's and research into the public health should be investigated. Future research should involve more in depth analysis of mechanisms of antibiotic resistance which is critical for developing any future effective plan for reducing the possible impact of ARB and ARGs on public and environmental safety.

| Location | Sampling Point | Temp. | Distance from Site* | Wind Speed | Sampling Time | Number of Samples |
|---|---|---|---|---|---|---|
| Site 1 ** 3/31/14 | Upwind Point A | 27.8°C | 74 m | 2.1 m/s | 10:56AM - 11:56AM | 2 |
| | Downwind Point A | 27.8°C | 69 m | 2.1 m/s | 11:01AM- 12:01PM | 2 |
| | Downwind Point B | 27.8°C | 80 m | 3.1 m/s | 12:13- 1:13PM | 2 |
| Site 2 ** 9/19/14 | Upwind Point A | 26.8°C | 541 m | 2.1 m/s | 9:31 AM/ 30 min | 3 |
| | Downwind Point B | 28.0°C | 127 m | 2.4 m/s | 10:12 AM/ 30 min | 3 |
| Site 3** 12/11/14 | Upwind Point A | 15.6°C | 29 m | 4.1 m/s | 1:00PM/ 30 min | 3 |
| | Downwind Point B | 16.1°C | 445 m | 2.6 m/s | 1:45PM/ 30 min | 3 |
| Site 4** 2/14/15 | Upwind Point A | 24.4°C | 77 m | 2.1 m/s | 3:00PM/ 45 min | 3 |
| | Downwind Point B | 23.9°C | 72 m | 1.5 m/s | 3:50 PM/ 45 min | 3 |
| Control 9/5/14 | Upwind Point A | 27.8°C | NA | 3.6 m/s | 2:21 PM/ 30 min | 3 |
| | Downwind Point B | 31.2°C | NA | 1.7 m/s | 1:31 PM/ 30 min | 3 |

**Table 4 - 1.** Locational information for each upwind and downwind sampling point

*Distance was measured from the sampling location to the nearest edge of the nearest CAFO building **The CAFOs tested for were surrounded by agricultural activities that did not involve animal production.

| Target | Primer | Concentration | Sequence (5'-3') | Annealing Conditions | Elongation conditions | Reference |
|---|---|---|---|---|---|---|
| *erm* (F) | ErmF-F | 500 nM | TCG TTT TAC GGG TCA GCA CTT | 60°C/30s | | |
| | ErmF-R | 500 nM | CAA CCA AAG CTG TGT CGT TT | | | |
| *bla*<sub>TEM</sub> | BlaTEM-F | 400 nM | TCG GGG AAA TGT GCG | 50°C/60s | 72°C/60s | |
| | BlaTEM-R | 400 nM | GGA ATA AGG GCG ACA | | | |
| *bla*<sub>SHV</sub> | BlaSHV-F | 400 nM | TGA TTT ATC TGC GGG ATA CG | 55°C/60s | 76°C/30s | |
| | BlaSHV-R | 400 nM | TTA GCG TTG CCA GTG CTC G | | | |

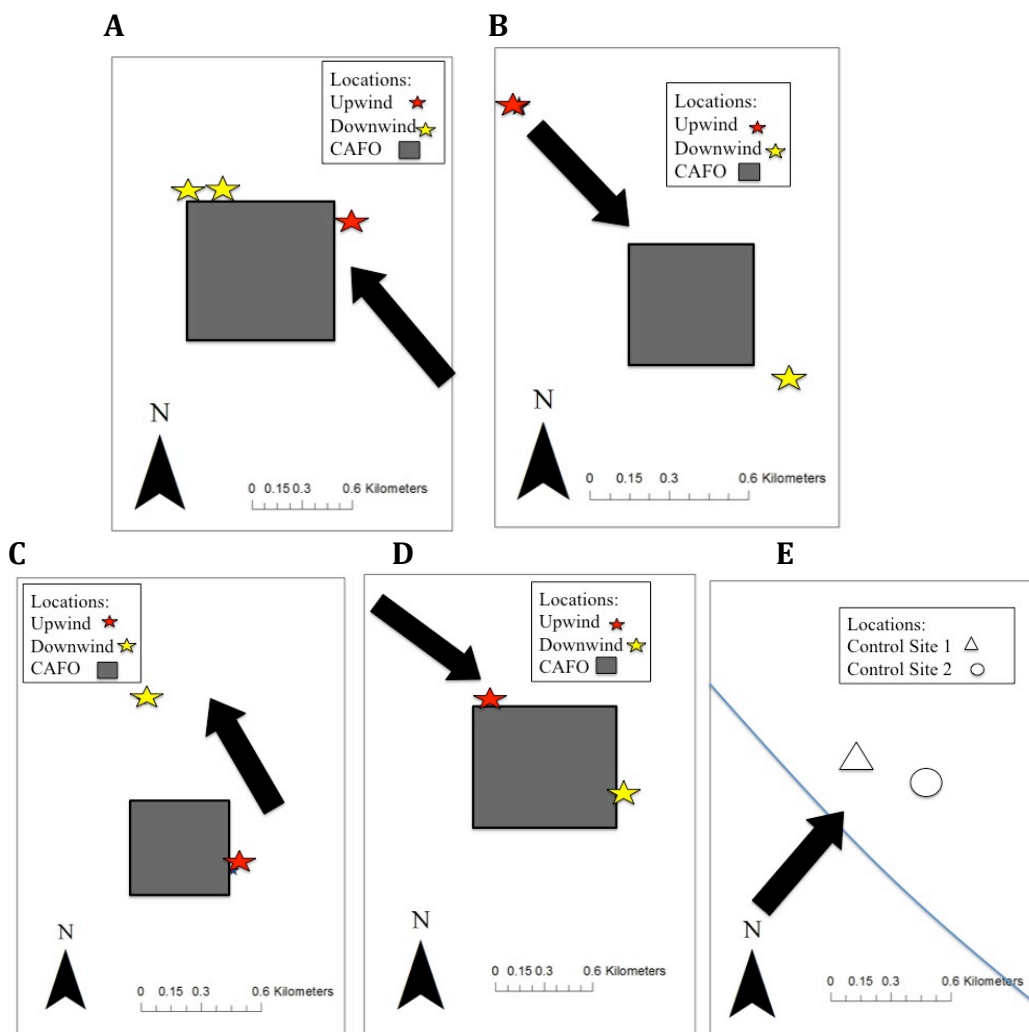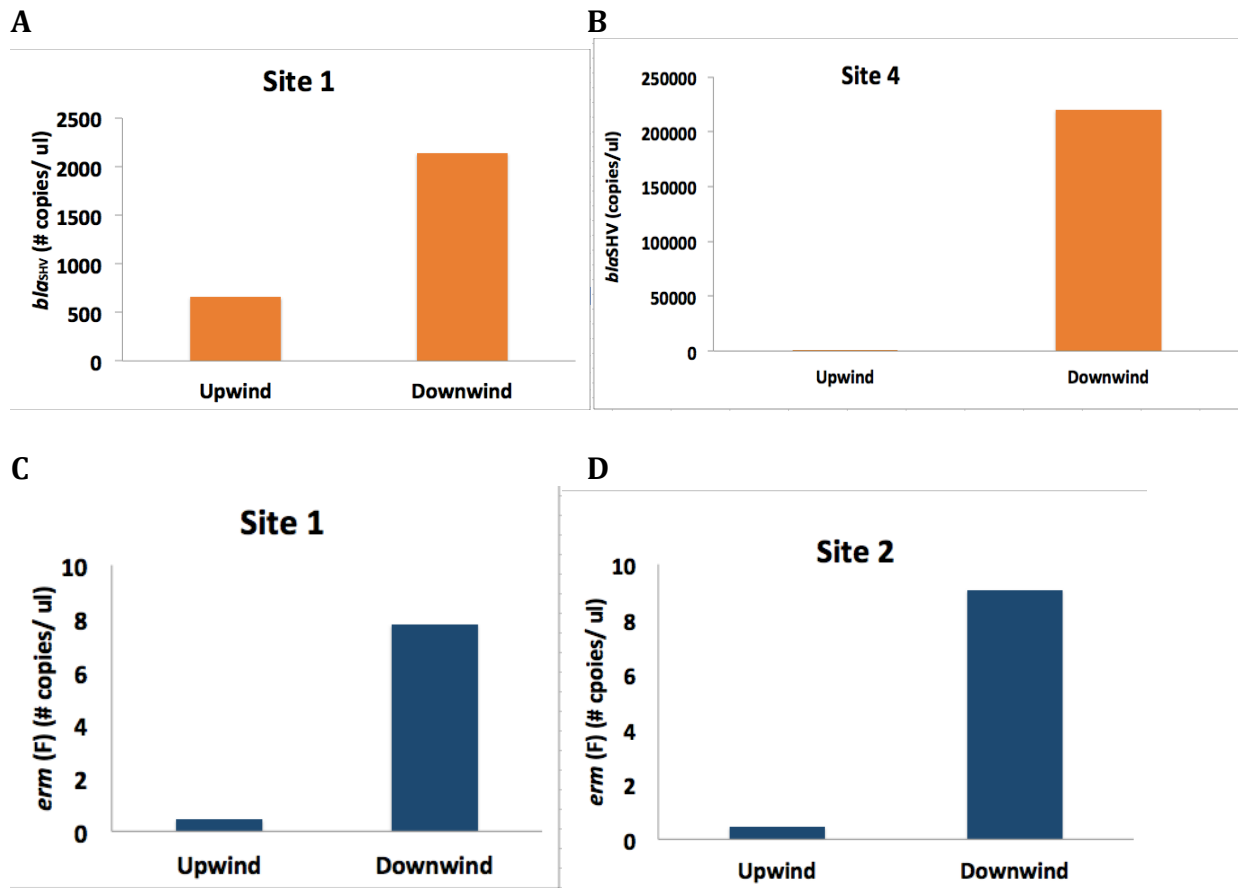**Table 4 - 2.** The primer sequences and qPCR reaction conditions used in the study

**Figure 4 – 1**. Maps of upwind and downwind sampling locations A) Site 1 B) Site 2 C) Site 3 D) Site 4 E) Control Site (blue line is beach/land frontier). Black Arrow indicates wind direction.

**A**



Site 1

**B**



Site 4

**C**



Site 1

**D**



Site 2

E
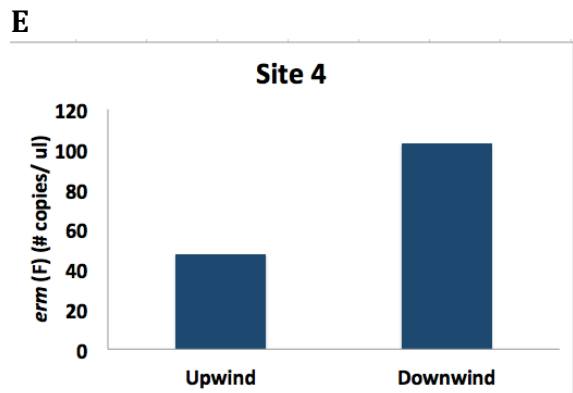


**Figure 4 - 2.** Resistance gene copy numbers of *bla*<sub>shv</sub> and *erm* (F) for upwind and downwind sites. A, B) *bla*<sub>shv</sub> for sites 1 and 4. B, C, D) *erm* (F) for sites 1, 2 and 4.

**Appendix C**

**Table 4 - 3.  DNA extraction for Control Site, and sites 2 and 3**

| Location | Sampling Point | DNA sample (triplicate average) (ng/uL) | Standard Deviation |
|---|---|---|---|
| Control Site (9/5/14) | Upwind Sampling Point | 7.92 | 0.87 |
| | Downwind Sampling Point | 6.18 | 0.80 |
| Site 2 Location (9/19/14) | Upwind Sampling Point | 1.66 | 0.36 |
| | Downwind Sampling Point | 1.97 | 0.57 |
| Site 3 | Upwind Sampling Point | 1.80 | 0.37 |

| Location (9/19/14) | Downwind Sampling Point | 1.46 | 0.40 |
| --- | --- | --- | --- |
| Site 4 | Upwind Sampling Point | 1.11 | 0.34 |
| Location (2/14/15) | Downwind Sampling Point | 1.09 | 0.29 |

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**Chapter 5. Summary: Antibiotic Resistance in Bacteria Isolated from Commercial Meat Samples and Air Samples Near Agricultural Sites**

Antibiotic resistance in bacteria is major public health concern as it can shorten the useful life of drugs on which we currently rely. There are still many questions regarding the role of the environment in the transport and proliferation of antibiotic resistant bacteria (ARB). The study done in Chapter 2 tested two hypotheses: 1) airborne bacteria near conventional beef cattle farms have higher resistance to six tested antibiotics when compared to bacteria near organic farms, using two growth modes 2) antibiotic resistance genes (ARGs) are detected at a higher frequency at conventional beef cattle farms when compared to organic farms. To our knowledge, ours is the first study to examine the air adjacent to cattle animal farming operations for either antibiotic resistance in viable bacteria or presence of ARG to compare production style in relevance to

antibiotic resistance. Our results show, consistently, that conventional beef production sites showed a greater average fraction of ARB than organic production for all six antibiotics at the low and high concentrations. Additionally, ARGs of $bla_{TEM}$, $bla_{SHV}$, $erm$(F) were observed at measurable levels within proximity to conventional farms, but not organic farms. Also, in many cases, isolates that would have been deemed susceptible when grown as a biofilm where then resistant in liquid culture, or vice versa. Because bacteria in different growth modes vary metabolically, this information could prove very important. Our results are also among the first qPCR data for ARG in air downwind of any agricultural site. Unlike previous work, this study expands upon current monitoring approaches by combining both culture based methods and molecular techniques to determine presence of ARB and ARGs from airborne bacteria. This novel sampling approach provides a unique framework for future studies that aim to test for presence of airborne ARB. This information is important to understand the potential exposure to ARB and ARGs via an air pathway and also for further studies to test for mechanisms of antibiotic resistance in airborne ARB and ARGs.

In Chapter 3, the study tested the hypothesis that there would be differences in antibiotic resistance among *Conventional*, *No Antibiotics* brands, and *Pristine Organic* brands. To our knowledge, this is the first study to test antibiotic resistant *E. coli* in several *Pristine Organic* retail meat sources. Our results showed that for four out of six antibiotics, the *Pristine Organic* poultry's fraction of resistant was significantly lower than that from meat from the *No Antibiotics* and *Conventional* categories. Our findings are consistent with the suggestion that the presence of antibiotic resistant *E. coli* differs depending on the type of poultry production system, and it is the first to suggest that a particular type of organic meat production shows a significant improvement in antibiotic resistance over typical organic brands. addresses a critical public

114

concern. This information is of wide interest and indicates a need for further studies in antibiotic resistance among *Pristine Organic*, *Conventional* and *No Antibiotics* categories.

In Chapter 4, we examined for antibiotic resistant genes of bioaerosols from upwind and downwind locations of poultry CAFOs To our knowledge, this is the first study to examine air samples collected upwind and downwind of CAFOs for elevated levels of ARGs. Results showed that ARGs are more abundance downwind of a poultry site than upwind in three of the four sites. Our study suggests that poultry CAFOs can be a point source of ARG environmental pollution. Livestock given antibiotics serve as breeding grounds for the emergence of new resistant strains of bacteria. The higher frequency of ARG detection near downwind versus upwind of poultry farms collectively show the occurrence of antibiotic resistance in the environment, which may imply potential exposure to ARGs via an air pathway downwind of poultry farm. Future research should involve more in depth analysis of the microbiome of family workers impacted by these ARG's and research into the public health should be investigated. This will be critical for evolving any future plan for reducing the possible impact of ARGs on the public and environment.