HERD-LEVEL RISK FACTORS ASSOCIATED WITH ANTIMICROBIAL SUSCEPTIBILITY PATTERNS AND DISTRIBUTIONS IN FECAL BACTERIA OF PORCINE ORIGIN

A Dissertation

by

SUSAN NOBLE ROLLO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2011

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Bo Norby
Committee Members,	Virginia Fajt
	H. Morgan Scott
	J.C. Huber
	Melissa Libal
Head of Department,	Evelyn Tiffany-Castiglioni

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ABSTRACT

Herd-level Risk Factors Associated with Antimicrobial Susceptibility Patterns and Distributions in Fecal Bacteria of Porcine Origin.

(August 2011)

Susan Noble Rollo, B.S.; M.S., Texas Tech University;

D.V.M., Texas A&M University

Chair of Advisory Committee: Dr. Bo Norby

The purpose of this dissertation is threefold: to determine the differences in apparent prevalence and the antimicrobial susceptibility of *Campylobacter* spp. between antimicrobial-free and conventional swine farms; secondly, to introduce an appropriate statistical model to compare the minimum inhibitory concentration distributions of *Escherichia coli* and *Campylobacter* spp. isolated from both farm types; and thirdly, to examine the potential herd level risk factors that may be associated with antimicrobial resistance of *Campylobacter* spp. and *E. coli* isolates from finishers on antimicrobial-free and conventional farming systems. In addition, a critical review of studies that have compared the levels and patterns of antimicrobial resistance among animals from antimicrobial-free and conventional farming practices was performed.

Fecal samples from 15 pigs were collected from each of 35 antimicrobial-free and 60 conventional farms in the Midwestern U.S. *Campylobacter* spp. was isolated from 464 of 1,422 fecal samples, and each isolate was tested for susceptibility to 6

antimicrobials. The apparent prevalence of *Campylobacter* spp. isolates was approximately 33% on both conventional and antimicrobial-free farms. The proportion of antimicrobial resistance among *Campylobacter* was higher for three antimicrobials within conventional compared to antimicrobial-free farms.

The susceptibilities of populations of bacteria to antimicrobial drugs were summarized as minimum inhibitory concentration (MIC) frequency distributions. The use of MIC values removed the subjectivity associated with the choice of breakpoints which define an isolate as susceptible or resistant. A discrete-time survival analysis model was introduced as the recommended statistical model when MICs are the outcome.

A questionnaire was completed by each farm manager on biosecurity, preventive medication, vaccines, disease history, and production management. Multivariable population-averaged statistical models were used to determine the relationships among antimicrobial susceptibility patterns and potential herd-level risk factors. Controlling for herd type (antimicrobial-free versus conventional), each antimicrobial-bacterial species combination yielded unique combinations of risk factors; however, housing type, history of rhinitis, farm ventilation, and history of swine flu were significant in more than one model. A variety of herd-level practices were associated with the prevalence of antimicrobial resistance on swine farms. Further studies are encouraged when considering interventions for antimicrobial resistance on both antimicrobial-free and conventional farms.

DEDICATION

To my niece, Ashley Rose Rollo

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I would like to thank my committee chair, Dr. Norby, and my committee members, Dr. Scott, Dr. Fajt, Dr. Libal and Dr. Huber, for their guidance and support throughout the course of this research. I would like to thank Dr. Norby for the opportunity to work on this research project. I would like to thank Dr. Scott for his influence in the field of antimicrobial resistance and epidemiology. I would like to thank Dr. Huber for guidance in biostatistics and public health epidemiology. I would like to thank Dr. Fajt for her technical expertise in the pharmacological aspects of antimicrobial resistance.

NOMENCLATURE

ABF	Antimicrobial-free
AMR	Antimicrobial resistance
CI	Confidence interval
DTSA	Discrete-time survival analysis
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MIC_{50}	Minimum inhibitory concentration that inhibits the growth of 50% of isolates tested
MIC_{90}	Minimum inhibitory concentration that inhibits the growth of 90% of isolates tested
m-PCR	Multiplex PCR

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

For more than 30 years, agricultural animal farming has been oriented toward highly structured processes that often involve multiple applications of antimicrobial drugs for the prevention, control, and treatment of disease and for the promotion of animal growth. However, consumers have become increasingly concerned about the presence of antimicrobial-resistant bacteria in foods of animal origin, and during the past decade there has been a significant consumer trend toward purchasing natural foods and supporting organic farming practices. Due to the rising demand for organic and antimicrobial-free animal products, some producers have voluntarily ceased to use antimicrobial drugs (Aarestrup et al., 2001a; WHO, 2003). Government agencies in some European countries have gone so far as to ban many of the antimicrobial drugs that are used for growth promotion (Aarestrup et al., 2001a; Grave et al., 2006). In the U.S., because of the rising concern about development, propagation, and accumulation of antimicrobial resistance, some drugs that are used for the treatment of disease have been withdrawn (examples include enrofloxacin and sarafloxacin for the treatment of disease in poultry) (Federal Register, 2001).

This dissertation follows the style of Preventive Veterinary Medicine.

Critics have suggested that there may be drawbacks to this trend (Singer et al., 2006). The voluntary or required cessation of antimicrobial drug use in farms may result in an increase in the prevalence of zoonotic pathogens, potentially increasing the risk to humans or negatively affecting animal well-being (WHO, 2003). For example, the ban on antimicrobial growth promoters in Denmark in the late 1990s was followed by an increase in therapeutic antimicrobial use in the treatment of *E. coli*–related disease (WHO, 2003; Grave, 2006). Hence, disease management and infection control are issues that antimicrobial-free producers have to address when they decide to avoid antimicrobial drugs. It cannot be assumed that simply eliminating antimicrobial drug use from an established production system will always result in safer food products.

There is a great deal of debate about the overall value of organic farming practices and the effects of eliminating antimicrobial drug use. Researchers who have tackled these issues have run into a morass of methodological problems and, in some cases, may have put forward conclusions that were unwarranted by the data. In this study we provide an analysis of the existing research literature on the effects of eliminating antimicrobial drug use in animal farming. We offer a detailed critique of the methodological issues that have plagued this research, and we show that in some cases highly suspect conclusions were reached. The result of our analysis is to describe protocols that can be used by future researchers to improve the validity of their comparative data.

1.1.1 Organic animal farming: Definition and debates

Organic farming is an expansive concept that can mean different things to different people. In the United States, the development and administration of organic farming standards is organized by the U.S. Department of Agriculture's National Organic Program (NOP). The NOP standards for animal farming specify that the production system cannot involve any use of hormones for growth encouragement or for antibiotic purposes (though vaccines are allowed and sick animals can be permanently removed from the production system for treatment). Additional requirements are also included in the NOP standards, such as the use of organic feed and a certain amount of outdoor exposure (USDA, 2010). Since the precise definition of organic farming can be hard to pin down, some researchers instead focus on the term "antimicrobial-free farming" (Baker, 2006). However, even the seemingly straightforward term "antimicrobial-free" can be defined in more or less stringent ways, as will be discussed below in subsection 1.2.4. While acknowledging that "organic" often has more expansive connotations, in this dissertation "organic farming" and "antimicrobial-free farming" are used interchangeably to refer to any animal production system in which the use of antimicrobial agents is prohibited. What it means to prohibit antimicrobial agents is a matter for detailed discussion in the following subsections.

The reasons for wanting to prohibit antimicrobial agents are relatively straightforward. Starting in the 1950s, antibiotics were increasingly used in both human and animal medicine (Aarestrup, 2006; Guardabassi and Kruse, 2008). In food animals, they were adopted for growth promotion as well as for the treatment, control, and

prevention of disease (McEwen and Fedorka-Cray, 2002). However, the broad use of antimicrobial drugs in animal farming created conditions in which antimicrobial-resistant (AMR) bacteria could emerge. Such bacteria were found to be accumulating in farm environments and then disseminating among different species and populations (Baquero and Canton, 2009). Researchers also found that antimicrobial resistance can spread among bacteria via the transmission of resistance determinants (genes) located on mobile genetic elements such as plasmids and transposons (O'Brien, 2002; Baquero and Canton, 2009). The significance of these mobile genetic units is that resistance can develop and spread much faster than would be predicted by basic evolutionary dynamics.

Most researchers assume that a gradual increase in the proportion of AMR bacteria is likely to occur after an antimicrobial drug is used for a prolonged period of time in an animal population under circumstances that allow for selective and evolutionary events to accrue over time. It seems reasonable to suppose that the prevalence of AMR bacteria is associated with or proportional to the route in which antimicrobial drugs are given, the total volume of antimicrobials used over time, and the nature of the treatment program (treatment of individual animals vs. treatment of entire populations). With the cessation of antimicrobial use in a population of animals, which is what occurs in the shift to organic farming, one would expect to see a decrease or possibly an elimination of resistant bacteria. Resistance to antimicrobials may incur a fitness cost (a decrease in the ability of a bacterium to compete with other bacteria in the environment) that would cause bacteria that acquire additional resistance genes to

become less fit (Andersson, 2003). Organic farming organizations, many researchers, and many regulatory policymakers have thus claimed that the cessation of antimicrobial drug use will (in addition to other benefits) result in more wholesome animal products with less chance of resistant bacteria ending up in the food supply. However, this has been disputed by many farming organizations and politicians, as well as by some researchers.

As a result of this controversy, a number of studies have been conducted to compare the relative proportions of resistant bacteria (from a variety of different bacterial species) in conventional farming systems versus organic farming systems. In addition, several review papers have been written on this subject (Jacob et al., 2008; Wilhelm et al., 2009; Young et al., 2009). However, these reviews have been wholly inadequate in reflecting the complexity of the data and in addressing methodological inconsistencies. In these reviews, authors failed to discuss the limitations in the existing literature that make comparison and summation difficult. (These limitations are described in detail in the second part of this section.) The existing reviews involved scientifically based, systematic selections of studies, but conclusions were summarized across studies in a way that was often improper and unjustified. For example, Jacob et al. (2008) acknowledged that the studies they reviewed did not all use the same definition (breakpoint) for determining whether bacterial isolates would be classified as resistant or susceptible. However, these reviewers did not elaborate on which studies differed in this respect or how the differences might affect comparability. Likewise, in Wilhelm and colleagues' (2009) study, the authors specifically pointed out that their objective was to

summarize results across studies using systematic review techniques, but they did not consider differences in sample sizes, nor did they discuss the different criteria that were used in various studies to select and define organic versus conventional farms.

1.1.2 Purpose of the study

Our main objective was to provide a rigorous review of previous studies in which levels and patterns of bacterial antimicrobial resistance were compared in organic versus conventional farming systems. We focus on issues that can make generalizations unreliable and that can cause difficulties for comparison and summation across studies. A review of this subject matter is very intricate due to the complexity of the data, and therefore, a thorough analysis is warranted. The value of this analysis is that it highlights problems with the existing research literature such that comparisons should be interpreted with caution. Standardization or consensus would be valuable for developing more reliable results which will help in making policy decisions, informing consumers, and for developing and designing future research.

1.1.3 Selection of relevant literature

Relevant literature was identified by searching major electronic bibliographic databases in June of 2009 and again in August of 2010. A matrix of key terms was used to search for studies in which farms that do not use antimicrobials were compared against conventional farms that do use antimicrobials (see Appendix A). The databases that were searched included Ovid (CAB, FSTA, AGRIS, and CAB), ISI (Web of Science), and PubMed (Medline). The years accessed were 1985 through 2010, and selections were restricted to English-language journals. Additionally, references from

review articles and other significant publications were checked and included in the data set where relevant. After reviewing the abstracts and titles of more than 200 relevant articles, 25 were selected for evaluation. The inclusion criteria were that any included study included farm animal populations and involved a comparison of bacterial antimicrobial resistance in organic farming systems versus conventional farming systems (see Appendix B for article details). We only considered studies that collected samples on the farm rather than in harvest or postharvest settings.

1.2. Problems in the Existing Research Literature

In reviewing the literature, we found that there are significant and systematic methodological factors in these studies that limit their usefulness for reaching comparative conclusions about levels of AMR bacteria in organic versus conventional farms. Some of the problems had to do with the internal validity of the studies, while others were related to their generalizability and to the possibility of making cross-study comparisons. In the following subsections, we break down these issues into seven specific problem areas. First, however, we will provide a brief introduction to the format of these studies. The unit of comparison in most of the studies was individual bacterial isolates from fecal samples that were collected from individual animals on organic and conventional farms. These bacterial isolates were usually analyzed for resistance to a group of several antimicrobials (the specific antimicrobials varied among the studies and depended on the types of bacteria that were being examined). An isolate may harbor a specific phenotype of resistance to a certain antimicrobial. However, *not* finding a

phenotype did not necessarily mean that it was not present in the animal. Also, a farm could be said to be "positive" in harboring a specific resistance phenotype if that phenotype were isolated from an animal on the farm. However, *not* finding a phenotype did not necessarily mean that it was not present on the farm. The working assumption in most of the studies was that the isolate would reveal the most dominant bacterial strain within the fecal sample of the animal that was selected.

1.2.1 Sample size

Interpretations concerning the prevalence of AMR bacteria on conventional and organic farms have been hampered by the limited number of samples collected and the limited number of isolates available for antimicrobial susceptibility testing. One of the major limitations for studies of specific resistance phenotypes is the number of the samples or animals included in the study. There can be a great deal of variation in the prevalence of the target bacteria and in the prevalence of resistant phenotypes among the target bacteria in a given animal population. In some studies, researchers investigated differences in AMR in commensal bacteria (e.g., *E. coli* or *Enterococcus* spp.), which will be present in all fecal samples, while in other studies they investigated differences in AMR in pathogens that are only present (or cultivable) from some animals (e.g., *Campylobacter* in poultry or *E. coli* 0157:H7 in cattle) . Furthermore, not all bacterial strains (be they commensal or pathogen) within a species will have a specific AMR phenotype or pattern. This may result in a very low power to detect differences among the populations that are purportedly being studied.

Sample-size determination is thus necessary in order to draw appropriate inferences when comparing the proportion of AMR bacteria present on different farms. In many studies, however, *a priori* sample-size calculations were not performed (or, at least, were not mentioned in the study reports). In some cases, the sample-size calculation *might* have been based on the estimated true prevalence of a pathogen, but since only a fraction of these pathogens carry resistance to specific antimicrobial drugs, the power to determine true differences in susceptibility in such studies may be very low. Compared to commensal bacteria, many pathogenic bacteria have a low prevalence in farm animal populations. These include *Listeria monocytogenes* (Schwaiger et al., 2009), Shiga-toxin *E. coli* (Cho et al., 2006; Cho et al., 2007; Reinstein et al., 2009), and Non-Typhi *Salmonella* (Siemon et al., 2007; Gebreyes et al., 2006; Ray et al., 2006). In one study, out of 799 cloacal swabs from chicken farms in Germany, only 12 *Listeria* isolates were obtained (Schwaiger et al., 2009). This small sample makes it difficult to meaningfully evaluate whether there are differences in the overall bacterial AMR on different farms. It is possible to use a *post-hoc* statistical technique to analyze the power of a study (that is, the likelihood that a difference would have been found if one was actually present). However, this was not done in any of the studies in the reviewed literature in which no differences were found between organic and conventional farms. Therefore, it is hard to know whether the findings of no difference were due to an actual lack of difference or were merely a result of having small sample sizes and insufficient statistical power.

1.2.2 Sampling selection

A second issue that makes it difficult to assess the results of previous studies is the variation in how subjects (animals) were sampled on the farms and how bacterial isolates were chosen to undergo antimicrobial susceptibility testing. For example, samples of milk were treated in three distinct ways. Some were pooled from a group of cows (Sato et al., 2004b), others were pooled from the four milk quarters of individual cows (composite milk samples, one sample per animal) (Bombyk et al., 2008), and some were taken as quarter samples and not pooled at all (four samples per animal) (Pol and Ruegg, 2007). The assumption when using pooled samples to determine pathogen prevalence is that if one animal is positive then the entire pool will be positive (Salman, 2003). Pooled milk samples can be informative when the expected prevalence is low and the objective is to determine pathogen endemicity. However, pooled samples are not as helpful in determining the relative prevalence of antimicrobial susceptibility on different farms, because each farm would normally have unique isolates within different animals on the same farm.

Another sometimes employed sampling method was selecting pathogenic bacteria from clinical or subclinical animals, as opposed to obtaining commensal bacteria (Docic and Bilkei, 2003; Garmo et al., 2010; Roesch et al., 2006; Bennedsgaard et al., 2006). This is a form of targeted sampling since the expected prevalence of the bacteria in the selected animals is higher than that in the overall population (Salman, 2003). One example of this approach is a study by Roesch et al. (2006), in which milk quarters from individual cows were sampled based on their reactivity to the California

Mastitis Test. This was done to increase the probability that pathogens would be present in the samples. While this is not an internal problem for Roesch and colleagues' study, it becomes a problem in research reviews when these results are used as a basis for comparison with other studies in which targeted sampling was not used. A comparison of data from studies that used targeted sampling against data from studies that did not use targeted sampling (Bombyk et al., 2007; Pol and Ruegg, 2007) is clearly inappropriate.

An additional issue that made comparisons of studies difficult was that in many of the reviewed studies, multiple colonies were selected from the culture plate of one sample/animal to help ensure that an isolate would be available for antimicrobial susceptibility testing (e.g., Gebreyes et al., 2006; Ray et al., 2006). In one study, the total isolate count actually came out to be greater than the number of animals used to determine animal-level prevalence (Ray et al., 2006). Increasing the number of selected isolates for susceptibility testing will increase the probability of finding low prevalence antimicrobial resistance, and it is a legitimate technique that is conducive to determining the presence of antimicrobial resistance (if the goal is to increase the chance of finding resistant bacteria, then obtaining many isolates per sample would be preferred). However, prevalence estimates from studies that sample more than one isolate per sample cannot be compared directly to other studies that sample only one isolate per animal.

In some of the reviewed studies (Heuer et al., 2001; Pol and Ruegg, 2007), only a subset of the total number of isolates were selected for susceptibility testing—in other

words, not all of the isolates that were obtained were tested. Heuer et al. (2001) sampled 10 animals from each of 160 flocks on 39 different farms, but selected only 53 of the resulting isolates for antimicrobial susceptibility testing. Reinstein et al. (2009) randomly selected an equal number of isolates from each production system. When only a subset of isolates is selected for testing, the power of the study and the precision of the prevalence estimates are decreased. Such studies have an increased chance of resulting in a Type II error (concluding there is no difference when there really is one). Additionally, the process of choosing a subset of isolates may introduce selection bias into the study if the selection is not performed in a random fashion. If the sample variance between different animals or within the same animal is not known, it will be difficult to determine whether to use frequency sampling to chose the isolates or whether to weight the sampling by the number of isolates available per animal. In Heuer's study, one would additionally need to know the partitioning of variance between farm, flock, and individual animals in order to determine a truly random sampling design among the available isolates. Furthermore, none of the study reports addressed these issues of random selection in the hierarchical structure.

In some of the reviewed studies, samples were collected from a large number of animals in order to better characterize antimicrobial susceptibility in herds (e.g., Villarroel, et al., 2006). In other studies, however, researchers focused on collecting multiple samples from a few animals in order to increase the sensitivity to detect the prevalence of rare resistant phenotypes (Dunlop et al., 1999). Sampling multiple animals enhances the ability to characterize between-animal and between-group variability. This

is particularly helpful since much of the variability in these kinds of studies can be attributed to the bacterial diversity within one animal. However, the value of collecting samples from a large number of animals is often specifically relative to the antimicrobial drug and bacterial species combination being tested and to the distribution of the variance within animals as compared to the variance between animals and between farms (Villarroel et al., 2006). For example, in looking at variation among cows, there may not be much difference in sampling multiple cows versus sampling multiple isolates per cow when it comes to determining variability (Villarroel, et al., 2006). Of course, the potential for clustering of AMR phenotypes within samples taken from only one animal or a few animals can produce additional limitations on the ability to determine the bacteria's overall prevalence (Berge et al., 2003 and Alali et al., 2008).

When considering many-animal versus few-animal studies, it must be concluded that there is no single best method for comparing AMR patterns among groups of animals (Wagner et al., 2002). However, the power of a particular study design in a particular context can be identified. If most of the variability is between-farms (as opposed to within-farm variance), then a study will have more power if more herds are sampled. Between-farm and within-farm variances are estimable in all studies where multiple animals on multiple farms are sampled. However, very few studies (excluding Dunlop et al., 1999, Wagner et al., 2002, and Villarroel et al., 2006) reported this breakdown of variance between and within farms. Thus, it can only be assumed that when most of the studies were conducted, no knowledge of the variance partitioning between and within farms was available to guide the sampling design.

The issue of how many farms to sample versus how many samples to select within farms is further complicated with the addition of several hierarchical levels within a farm. For example, to estimate the most efficient sampling design on a pig farm, one would need estimates of variance at the herd-level, the house-level, the pen-level and the level of individual pigs within a pen. If several different production groups within a farm are sampled, then the variance among the production groups may also need to be accounted for. These variances may not always be significant—for example, Dunlop (1999) determined that most of the variance in a study on pigs originated from between individual animals and not between pens or buildings. Without an analysis of the variance, however, it is impossible to know whether or not the variances are significant, and thus it is impossible to know the relative power of these studies when it comes to the likelihood of finding differences in prevalence of AMR that may exist between organic and conventional farms. In some of the studies the power to find differences between farms may have been relatively high, and in other studies the power to find differences may have been relatively low. These differences should be taken into account when comparing and summarizing the findings from various studies; however, given the methodological limitations described above, it is simply impossible for us to know with any precision which studies had a lower power to find differences and which studies had a higher power to find differences.

Although different sampling methods can be justified under different circumstances, a standard format among comparative studies would be highly desirable. To maximize the ability to determine variance between farms, it is usually best to sample

larger numbers of animals from multiple farms. Of course, this may be difficult to accomplish given the relatively small number of organic farms and the logistical problems with sampling multiple farms on a large scale. More importantly, to allow for comparability, calculations of variability and study power should be conducted. The direct quantification of the absolute or relative number of bacteria that are resistant to a particular antimicrobial drug (using a medium that incorporates the antimicrobial drug in the agar plate) would be a preferable method for determining differences in resistance among farm types. This approach is tedious and very costly, however, so it may not be possible in many cases. Hence, for the purposes of determining patterns of antimicrobial susceptibility, sampling at the individual animal level with ideally one bacterial isolate per animal would be the best common protocol for establishing antimicrobial susceptibility patterns across studies.

1.2.3 Farm selection

A third issue that makes it very difficult to establish generalizations across the reviewed studies is that the methods used to select farms for participation varied tremendously. In some geographic areas there are relatively few organic farms, leading researchers to include a smaller number of organic farms than conventional farms in their comparative studies. For example, Nulsen (2008) sampled a total of four farms, but only one of them was an organic farm. The length of time that this farm had used organic practices was unspecified. In contrast, Garmo et al. (2010) invited all of the organic farms in Norway to participate in their study leading to a much better balance between conventional and organic farms in the final sample. These researchers also

matched conventional farms to organic farms by geography, housing system, breed, and herd size, thus removing these factors as potential confounding variables.

Convenience sampling of farms was a common practice in the reviewed studies (Sato et al., 2004a, 2005; Halbert et al., 2006; Ray et al., 2006; Bunner et al., 2007). The logistics of selecting organic farms in a random fashion (for example, from a list providing by the NOP) are often practically and economically unfeasible. Therefore, probability sampling of farms was not used in most of the studies, leading to a greater chance that an unrepresentative sample of farms may have been obtained. Convenience sampling can lead to a variety of problems—in one study, samples from farms in two countries (the U.S. and Denmark) were included for comparison, even though the samples from these different farms appear to have been obtained using different methodological protocols (Sato et al., 2004b). These are limitations that can lead to bias, and they should be mentioned as limitations when reporting comparative results.

The best practices for comparative studies would be to sample the same number of animals on each farm, using the same protocols, and to eliminate as many potential confounding variables as possible. Farms should be selected based on their representation of the target population and a method of random selection should be used if feasible. Since fewer organic farms are available, they should be selected first, and then conventional farms can be selected based on geographical proximity to organic farms and in such a way that eliminates confounding variables. Although matching by herd size would be preferable, this is typically not possible because organic farms in general are smaller than conventional farms. Researchers should faithfully record their

study limitations; reviews of the literature should reflect these limitations, and caution should be exercised when making generalizations and comparisons on the basis of studies that may contain sampling bias.

1.2.4 How an antimicrobial-free population is defined and selected

In the studies included in this review, the definition of organic and antimicrobial-free farms varied significantly. In other words, exposure (for both organic and conventional) was not comparable between studies. One common difference was whether or not organic farms would allow antimicrobial drugs to be given to a sick animal, which was then left in the herd while discarding the product (e.g., the milk) for a period of time. This is a common practice in Europe—whereas in the U.S. sick animals on organic farms are permanently removed from the herd for treatment (IFOAM, 2010; USDA, 2010). In one study, antimicrobial use was allowed on an "antimicrobial-free" farm in cases of calves with severe diarrhea or pneumonia (Sato et al., 2005). In another study, three treatments per year with antimicrobials were allowed for each individual cow on an organic farm (Garmo et al., 2010). In addition to this variation in antimicrobial usage, products used on particular farms can have an effect on selection for certain resistance mechanisms. For example, some of the organic farms that were sampled in the reviewed studies allowed the use of phytogenic feed additives. These are plant-derived growth promoters that have antimicrobial activity and therefore can co-select for antimicrobial resistance (Roesch et al., 2006). Some of the organic farms that were sampled may have used heavy metals, such as copper or zinc, in feed to help with growth promotion; researchers have found that these heavy metals may also have

antimicrobial activity (Hasman et al., 2006). Variations in such practices among the organic farms in the various studies could reasonably be predicted to cause variations in bacterial antimicrobial resistance patterns (Baker-Austin et al., 2006). Furthermore, some of the comparative studies defined "antimicrobial-free" farms only on the basis of whether or not antimicrobials were used prophylactically, and in one of these studies the authors did not specify whether or not antibiotics were used on the farms for other reasons (Docic and Bilkei, 2003). These discrepancies in the definition of antimicrobial-free farms make any comparison across studies very difficult, to say the least.

In addition, some researchers have used the "organic" label for farms that raise animals entirely on pasture (Siemon et al., 2007). Although being raised on pasture is not the same as being raised under organic practices, a recent review by Jacob et al. (2008) inappropriately categorized farm conditions as "organic" when it involved the exclusive use of pasture. One example of why this is problematic is that pasture-raised animals are likely to be exposed to significantly greater amounts of environmental *Staphylococcus aureus* (SA), which has been shown to be mostly novobiocin-sensitive, coagulase-negative *Staphylococcus* spp. (NSCNS). In contrast, animals in confinement under organic practices are more likely to have been exposed to novobiocin-resistant *Staphylococcus* spp. (NRCNS) (Matos et al., 1991).

One of the research practices that has led to discrepancies in the definitional parameters for organic versus conventional farms is the reliance on organic certification labeling from co-ops, producers, or government agencies. The farming practices required for such labeling vary greatly among different organizations. In most of the studies

under review, researchers identified organic farms based on the labeling of a co-op or similar regional organization. Even national organic labeling requirements, however, can vary between countries. One example of these international differences is the requirement for the use of organic feed. The International Federation of Organic Agriculture Movements, which is located in Germany and organizes much of the organic labeling in Europe, currently requires that 50% of the feed must be produced on an organic farm and that 60% of the total diet for ruminants must be roughage (IFOAM, 2010). This is in contrast to the NOP in the United States, which mandates different standards in regards to the origin and proportions of feed (USDA, 2010).

In recent years the organic industry has made progress in creating consistent international standards of practice. However, most of the studies under review were conducted in the early 2000s, when there were even greater differences in national and local policies for organic labeling requirements. Researchers should not consider these definitional inconsistencies to be a thing of the past. As recently as 2009, a review that compared organic and conventional dairy farm practices suggested that the standards in the United States were more stringent than those in other countries (Ruegg, 2009). For the sake of making consistent and legitimate comparisons, researchers should provide a specific account of what types of products and practices are included in their definitions of organic farms. This should include information concerning the use of ionophores and other feed additives.

The number of years that a farm has used organic practices is another factor that should be specified in comparative studies. It is plausible that the resistance levels to at

least some antimicrobial agents may be different depending on the number of years that antimicrobial agents have not been used in herds. Hence, the number of years that a farm has been antimicrobial-free should be investigated as a possible determinant in statistical models comparing resistance on organic and conventional farms. In addition, some organic farms change to antimicrobial-free practices after using conventional practices for a number of years, while other organic farms obtain new genetic stock from a variety of sources or exposures. Furthermore, the environments where organic farms are located may contain reservoirs of resistance genes. None of these issues were considered in the studies included in this review. It is a critical limitation in comparative studies that researchers failed to identify and recruit farms at the same number of years of being antimicrobial free to ensure that the exposure information pertained to an etiologically relevant time period.

1.2.5 Methods used to determine and report antimicrobial susceptibility

Phenotypic antimicrobial resistance is most often determined by exposing a bacterial isolate to increasing antimicrobial concentrations, by the use of *in vitro* tests, and measuring its survival on a gradient. The *in vitro* concentrations at which bacteria survive are then compared to benchmarks that signify clinical efficacy. In the U.S., these benchmarks are set by the Clinical and Laboratory Standards Institute (CLSI) to reflect a level of resistance that is likely to compromise the efficacy of antimicrobial treatment in an infected animal or human (CLSI, 2010). Based on susceptibility testing, bacteria are commonly divided into susceptible, intermediate, and resistant categories (or just divided into a susceptible / resistant dichotomy). The susceptible category includes

isolates for which the antimicrobial activity is associated with a likelihood of therapeutic success when the recommended dosages for a specific antimicrobial agent are used. The resistant category includes isolates for which the antimicrobial activity is associated with a higher-than-expected likelihood of therapeutic failure (CLSI, 2010; Kahlmeter et al., 2003).

According to the CLSI (M100-S19) (CLSI, 2010), the resistant and susceptible designations are determined by the breakpoints, which are specific levels of antimicrobial concentration (MIC) that inhibit bacterial growth because of resistance genes in the bacterial isolate. Also, breakpoints predict an outcome for a specific pathogen, in a specific disease, in a host species, given a particular regimen (i.e. dose, frequency, route, and duration) (CLSI, 2010). Some pathogen/drug combinations have an intermediate breakpoint, some have a susceptible and resistant breakpoint, and some have only a susceptible breakpoint when resistance to an antimicrobial drug has not yet been identified. Above and beyond the breakpoint MIC, a sample is considered to be meaningfully different from wild-type bacteria, in other words, to be a resistant strain (MacGowan and Wise, 2005). Clinically, breakpoints divide a population of bacterial isolates into those that are more likely to be susceptible to treatment and those that are more likely to be resistant to treatment. If breakpoints are too conservative, borderline susceptible bacteria may be considered fully susceptible, rather than partially resistant (Dalhoff et al., 2009).

Using microbiological breakpoints to categorize samples into a simple susceptible/resistant dichotomy may limit researchers' ability to compare susceptibility,

especially in cases where very few of the samples are classified as resistant. Using the simplified dichotomy does not always reflect the actual spectrum of resistance that exists in the samples. In addition, there is no gold standard for defining the breakpoints. The specific concentrations that define a resistant sample may vary in different countries (the CLSI's counterparts in Germany and the Netherlands use slightly different definitions) (GENARS, 2004; Schwaiger et al., 2009; MARAN 2004; Hoogenboom et al., 2008) and Europe as a whole has a separate standard (EUCAST). Another limitation is that the CLSI or other agencies may not have determined breakpoints for some bacterial–antimicrobial combinations (e.g., *E. coli* and ceftiofur) (Cho et al., 2007). Therefore, some of the studies under review used alternate sources for breakpoints. Some substituted human medical literature for veterinary standards (Pol and Ruegg, 2007; Ray et al., 2006). The standards for animal isolates and human isolates are established separately and, in theory, should not be used interchangeably in this manner. However, if the breakpoints were not established for animal isolates then authors had no choice but to use breakpoints from human standards or animal standards from different species.

Another alternative source of breakpoints in some studies was the National Antimicrobial Resistance Monitoring System (NARMS) (FDA, 2008). For example, Sato et al. (2004a) used NARMS breakpoints for *Campylobacter*, since no CLSI standards had been established for this bacterium. However, NARMS only had breakpoints for *C. jejuni* and not for other variants such as *C. coli*. In Sato et al's study, 30% of the isolates failed the hippurate test, indicating that the speciation was something other than *C. jejuni* (Sato et al., 2004a). Furthermore, the approved method of testing

Campylobacter susceptibility was not established until May of 2002, when NCCLS, the National Committee for Clinical Laboratory Standards, now the CLSI, published M31-A2 (NCCLS, 2002). M31-A2 clearly states that agar dilution is the method of choice for testing *Campylobacter* in relation to their breakpoints. However, Sato et al., 2004a used a different testing method, disc diffusion, and still other methods such as microbroth dilution have been used in later studies (Halbert et al., 2006). In summary, comparisons between studies can be greatly hampered by differences in the testing methods used and in the definition of breakpoint concentrations.

In many of the studies under review the breakpoints were not directly reported. However, the articles frequently referenced breakpoint sources, including CLSI publication M31-A2 (Gebreyes et al., 2006; Bombyk et al., 2008; Ray et al., 2006; Roesch et al., 2006; Hoogenboom et al., 2008), CLSI publication M2-A6-7 (Nulsen et al., 2008; Ray et al., 2006; Roesch et al., 2006), and CLSI publication M100-S10-12 (Nulsen et al., 2008; Ray et al., 2006). There are a few exceptions from the CLSI standards, including studies of ceftiofur and streptomycin that were based on NARMS breakpoints (Ray et al., 2006). In the studies under review here, significant breakpoint variations were not noted. However, researchers should understand that breakpoints can change as new information is obtained and new forms of resistance develop within bacteria (CLSI, 2010; specifically, see ceftriaxone reset breakpoints from earlier CLSI). The recommended practice is to use the standards of microbiological methodology and designated breakpoints from the most recent CLSI publication, and to report these methods and breakpoints explicitly in research articles. To facilitate comparisons

historically, the need for reporting of MIC values, in addition to breakpoint interpretations, is therefore readily underscored.

Beyond the issue of what breakpoint definitions are used, the results of antimicrobial susceptibility testing can be reported in a number of ways. The most common and straightforward way, as described above, is to divide the samples into resistant, intermediate, and susceptible categories following the breakpoints of the day. In studies where this method of reporting was used, there was a great deal of discrepancy in how isolates that fell in the "intermediate" category were classified. In some studies, isolates that fell in the intermediate category were consolidated into the resistant category (Pol and Ruegg, 2007; Roesch et al., 2006). In other studies they were consolidated into the susceptible category (Sato et al., 2005; Luangtongkum et al., 2006). Furthermore, while some researchers interpreted an isolate as resistant if its minimum inhibitory concentration (MIC) was greater than the listed breakpoint (e.g., Heuer et al., 2001), most interpreted an isolate as resistant if its MIC was *equal to* or greater than the breakpoint. Thus, there is a discrepancy in how the isolates that have an MIC equal to the breakpoint were treated.

Another way to report the resistance of bacteria in a group of isolates is to use the median level of resistance (median MIC; MIC_{50}) (Reinstein et al., 2009) or the mean level of resistance (mean MIC) (Docic and Bilkei, 2003; Mathew et al., 2001; Schwaiger et al., 2008, 2010). These techniques are applicable when all of the bacteria samples are being tested for susceptibility to a single antimicrobial agent. When the median MIC is used for comparison, the median values in two samples could be the same while the

actual MIC distribution differs. This is because the data are right censored and the group of isolates that did not exhibit growth inhibition at the highest dilution were classified as being equal to the highest dilution; when in reality, inhibition could truly occur at concentrations greater than the highest dilution. A statistical difference may be reported if one herd type had more values in the censored category, even when the median is the same for both herd types (Reinstein et al., 2009).

In some studies, the mean MIC ($\log_2$ transformed) was compared by t-test (Schwaiger et al., 2008; Docic and Bilkei, 2003; Mathew et al., 2001). The interpretation of this by one author was that the mean MIC described the prevalence and susceptibility (Docic and Bilkei, 2003). Reporting a mean differs from using a median, because the median divides the distribution in such a way that 50% of the isolates fall above and 50% below a given dilution (or discrete category), whereas the mean is just the average of all MIC values for each group of isolates. Moreover, the median does not depend on the nature of the underlying MIC distribution; be it unimodal or bimodal, for instance. A problem that emerged in some of the reviewed studies that reported a mean was that isolates in the dilution that were greater or equal to 256 µg/mL were designated as just being equal to 256 µg/mL, or in other words, were right-censored. In these cases, it has to be understood that the mean estimate is lower than the true mean and that the true value of the mean cannot truly be defined. Furthermore, in studies that reported the mean MIC, the breakpoints used for each of the antimicrobials tested were unspecified in the research reports (Docic and Bilkei, 2003; Mathew et al., 2001, Schwaiger et al., 2008, 2010).

Another variation in reporting when testing for susceptibility to one antimicrobial in a group of isolates from different farming systems was the use of MIC_{50} and MIC_{90} as alternative descriptive values (Soonthornchaikul et al., 2006; Bunner et al., 2007; Halbert et al., 2006). The MIC_{50} is the drug concentration that inhibits the growth of 50% of the isolates tested, while the MIC_{90} is the drug concentration that inhibits the growth of 90% of the isolates tested. The MIC_{90} was also used as a breakpoint when isolates fell below the threshold values designated by DANMAP, the Danish Integrated Antimicrobial resistance Monitoring and Research Programme (Sato et al., 2004b).

The implication of using different breakpoints and different methods in describing levels of bacterial resistance is that it greatly impedes our ability to make generalizations from the literature. When different studies report different kinds of measurements in their comparisons of organic versus conventional farms (e.g., using MIC_{50} or an MIC distribution), these results cannot be easily regarded as equivalent for the sake of making generalizations—even though some of the existing reviews attempt to do just this (Young et al., 2009; Wilhelm et al., 2009; Jacob et al., 2008). Summarizing across studies that use different outcome measures is neither appropriate nor justified in regards to making a broad inference based on individual study results. As an alternative to comparing the proportions of resistance in two or more populations, review studies could try to compare the full distribution of MIC values in these bacterial populations. However, the comparison of MIC distributions is inherently more complicated than comparing, for example, two different proportions that were reported in existing analyses. Some of the studies included in this review included a full MIC

distribution for their data (Sato et al., 2004b, 2005; Mathew et al., 2001; Ray et al., 2006). In some cases, differences between the MIC distributions of each herd type were measured rather than using mean, median, or MIC_{50} (Sato et al., 2004a), and in other cases the proportion within each MIC dilution was also reported for each bacteria (Thakur and Gebreyes, 2005; Bunner et al., 2007). By using the entire MIC distribution as an outcome measure as well as the proportion within each dilution, the assumptions inherent in using a breakpoint are eliminated and historical comparisons are more readily made, as standardized breakpoints change: now and into the future.

1.2.6 Methods used to isolate bacteria

Even if the full susceptibility data are available for cross-study comparisons, additional concerns about inherent microbiological limitations and potential biases in study design can hamper our ability to make generalizations across studies. For example, there is no single standard for *Campylobacter* isolation (Silley, 2003). When dealing with this bacterium, Sato et al. (2004a) did not use an enrichment media, as is commonly done by other researchers. Furthermore, Sato et al. used an incubation temperature of 37°F, rather than the standard 42°F, thereby discouraging thermophilic *Campylobacter* species such as *C. jejuni*, *C. coli*, and *C. lari*. A similar methodological divergence was made by Mathew et al. (2001), who used an enrichment broth to obtain more isolates of *Salmonella*.

A related methodological problem was that some researchers did not make distinctions between different subspecies of bacteria—for example, Heuer et al. (2001) did not differentiate *Campylobacter* species isolates into *C. jejuni* and *C. coli*. This may

have produced biased results, because each subspecies has been shown to exhibit different patterns of resistance (Moore et al., 2006). Erythromycin resistance has been shown to be rare in *C. jejuni* but common among *C. coli* strains, particularly among isolates from pigs (Moore et al., 2006; Harrow et al., 2004). The higher frequency of resistance of erythromycin in pigs has not been fully explained; however, Aarestrup and Engberg (2001b) suggested that it may be due to a generally higher frequency of mutations conferring resistance among *C. coli* or more selective pressure from prior use of antimicrobial agents. Likewise, *Enterococcus* species (*E. faecium and E. faecalis*) differ in susceptibility because *E. faecalis* is known to carry a unique, natural innate resistance to virginiamycin (Delgado et al., 2000). This lack of differentiation between subspecies greatly decreases the comparability between the results of different studies. It would be desirable for standard microbiological methods, as stipulated in the current publications of CLSI, to be used across all studies. This would improve comparisons across studies and reduce or prevent any biases that may occur in the laboratory.

1.2.7 Statistical analysis

In many of the studies that were reviewed, results were reported based on inappropriate statistical models. Ideally, the chosen statistical model(s) should reflect the nature of the data and account for assumptions or characteristics of the minimum inhibitory concentration frequency distribution. There are three major discrepancies in the statistical models used in the reviewed literature. First, many studies limited their analysis to reporting proportions without adjusting for the hierarchical nature of the data (Roesch et al., 2006; Gebreyes et al., 2006; Halbert et al., 2006). The hierarchical nature

of the data (individual animal level, production unit level, farm level, etc.) results in isolates from individual animals forming clusters. Clustered data should be accounted for in a statistical model in order to obtain valid estimates and appropriate standard errors (Dohoo et al., 2003). The underestimation of the standard error could result in researchers reporting differences that are not truly present (Type I errors). Using a statistical model that accounts for clustering will result in a more appropriate standard error measurement, which will likely be greater than the one found when using generalized linear models and not adjusting for herds.

There are two types of statistical models that can be used to explain dependency between animals (i.e., clustering). One is a subject-specific model that includes a random effect for each cluster (e.g., for each farm; or animal, if multiple isolates are tested) in the linear predictor of the model. A subject-specific model should be used if the goal is to interpret the results at the farm (animal) level. The second type of model that could be used is a population-averaged model, which involves using the expected values for a particular set of predictors averaged across the population of clusters. This would allow inferences to be made across all herds (animals) (Dohoo et al., 2003). Although several of the studies that were reviewed did account for clustering in the analysis (e.g., Bunner et al., 2007), most did not (e.g., Schwaiger et al., 2008, 2010; Cho et al., 2007; Gebreyes et al., 2006). One example of this is Sato et al. (2005), who did not account for dependency among animals within herds but rather used a simple logistic regression which assumes independence between animals. These authors stated that they avoided the issue of clustering by only obtaining one isolate per animal, but this does not account

for the dependence that would be expected between animals from the same farm. This inadequacy was corrected in an additional study (Sato et al., 2004a), in which the authors examined a different bacterium, *Campylobacter*, on the same farms as the previous study. Here, the researchers accounted for clustering and used a generalized estimating equation for the chi-square test (population-averaged).

The second major statistical discrepancy in the literature arose when the outcome of isolate susceptibility was reported as MIC distributions. Most of the researchers who did this failed to consider the censored nature of the data. Antimicrobial susceptibility tests such as agar dilution or microbroth dilution only have a set number of dilutions, concentrations, or categories, and in some cases there are only two to four dilutions around a breakpoint. Due to cost issues, increasing the number of dilutions may compromise the number of antibiotics that can be tested on a 96-well microbroth dilution plate. Any isolates that are not inhibited up to the highest dilution will be grouped in the highest category. For example, the graphs in Figure 3.1-3.6 illustrate the difference in distributions when various isolates are categorized in the highest dilution. Because of this, comparisons of left- and/or right-censored distributions should be done using a method that accounts for the censored nature of the data. Survival analysis (SA) is a statistical method that can be used when analyzing MIC distributions. SA, which is often considered for time to event data, can be used if the sequential dilutions of the test are interpreted as the time variable and SA is clearly favored when dealing with censored data. In the majority of the studies that were reviewed, the researchers did not consider the isolates in the highest dilution as censored when reporting and comparing MIC

distributions (this is reflected in the studies that mistakenly reported a mean MIC, as described previously). However, in a few studies researchers did consider the outcome data as a censored distribution and used SA for the analysis (Reinstein et al., 2009; Pol and Ruegg et al., 2007). The Wilcoxon test was used in some cases to determine the difference between herd type based on median MICs (Reinstein et al., 2009; Pol and Ruegg et al., 2007); however the Wilcoxon test does explicitly account for censoring.

The third major problem with the statistical methods used in the reviewed literature is that some researchers attempted to account for censoring by using a Cox model (also called a continuous-time proportional hazard model), but this model is not appropriate for such a task. The Cox model is popular and easily accessible in most statistical software programs. Continuous time models such as the Cox model make the assumption that an event (outcome) occurs at an exact moment in time. Instead, MIC data are measured discretely, by dilutions or concentrations where bacterial growth is inhibited. When two events occur at the same time, these are referred to in the Cox model as tied outcomes. If there are an excessive number of tied outcomes, then the Cox model will often fail (Willett and Singer, 2003). A study by Ray et al. (2006) is an example in which the Cox model was used to account for right-censoring while comparing organic and conventional farms. Farms were classified based on the highest MIC recorded among the isolates tested from that farm. The isolates with an MIC value in the highest dilution were considered censored; however, there were likely a large number of tied outcomes. The Cox model is inappropriate for data such as with MICs because of the number of tied outcomes involved (Cox models also involve an

assumption that the predictor does not change over time; more details about the use of such models is provided in Section 3). An alternative model that could be used for similar purposes is a discrete-time SA model as derived by Willet and Singer (2003). Such a model would be more appropriate because it would allow for discrete outcomes and would let the researcher take into account the censoring of the distribution.

An additional statistical consideration that should be taken into account is the systematic differences between organic and conventional farms. In general, organic farms are notably smaller. Herd size should therefore be checked as a potential confounder and should be included in the statistical model. If possible, factors such as age group or season should be considered for inclusion in the statistical model (Sato et al., 2004a, 2005). Another potential discrepancy that can arise in these studies is in regard to the type of covariates included in the model, that is, whether the covariate is measured at the animal level (e.g., weight, age) or at the herd level (e.g., herd size, season). The use of covariates may differ between studies, and this will alter the results and lead to a bias when making cross-study comparisons.

There are also inherent limitations in the reviewed studies in that most of them are cross-sectional studies. To our knowledge, there have not been any longitudinal studies conducted to compare the proportion of bacterial antimicrobial resistance in organic versus conventional farms. One reason for this is that longitudinal studies are time-consuming and costly. Making inferences based on cross-sectional studies should be done cautiously, because sampling in a cross-sectional study is a snap-shot of a particular time frame. Cross sectional studies do not allow researchers to determine rates

or to determine whether the exposure or the outcome occurred first. Cross-sectional studies are also subject to selection misclassification. In regard to sampling, small sample sizes favor the process of generating hypotheses rather than the process of testing them. In food animal production, cohort studies or hybrid cohort studies are the preferred type of observational study to provide validity in inference (even though these kinds of studies are difficult to implement). In a well-designed cross-sectional study, the test population should reflect the target population. This task is difficult to accomplish; however, considering the logistics of sampling multiply randomized farms, followed by a randomization sampling format within the farm environment.

1.3. Summary

Overall, our conclusion from this literature review is that caution should be used in interpreting previous studies in which antimicrobial susceptibility was compared in organic versus conventional farms, due to a number of inherent limitations and potential biases in these studies. The small number of isolates that were available for testing, the variations in how farms were selected and how isolates were collected, and the procedural problems in measurement and statistical analysis all indicate that generalizations and cross-study comparisons cannot be readily made from this literature. While we found that more studies suggested a greater prevalence of antimicrobial resistance on conventional farms, some studies suggested the opposite, and the prospect of reaching any definitive conclusion on the basis of the existing literature seems extremely unlikely. Presently, each bacterial species and each study should be evaluated

on an individual basis, and hopefully a consensus will become more likely after future studies in which attention to future comparisons is included at the design stage. There is a need for the interdisciplinary development of common protocols for quantifying resistance within and between bacterial and host populations, including laboratory methodologies and sampling designs in animal populations (Davison et al., 2000).

In the following subsections, we examine these methodological issues in even greater detail, with a particular focus on the format of the data. We make recommendations for common protocols that can be used in future studies to allow for a greater possibility of cross-study comparison and generalization. In Section 2, we use a population averaged model to account for the hierarchical nature of the data. In Section 3 we address the issue of MIC frequency distribution and present a new model that accounts for characteristics of a MIC distribution such as censoring and discrete outcomes. In the fourth section we consider whether particular risk factors can potentially be associated with the proportion of bacterial antimicrobial resistance on conventional and organic farms; this is a subject that is not adequately discussed in the current literature. The final section provides a summary and conclusion.

2. PREVALENCE AND PATTERNS OF ANTIMICROBIAL RESISTANCE IN *Campylobacter* SPP. ISOLATED FROM PIGS REARED UNDER ANTIMICROBIAL-FREE AND CONVENTIONAL PRODUCTION METHODS IN EIGHT STATES IN THE MIDWESTERN UNITED STATES*

2.1. Introduction

Campylobacter spp. are one of the most common causes of human diarrheal illness in the United States (Mead et al., 1999). Although most cases of campylobacteriosis are self-limiting, treatment with antimicrobial drugs is required in more severe or recurrent cases. The first and second most commonly identified subspecies that cause enteritis in humans are *Campylobacter jejuni* and *Campylobacter coli,* respectively (Tam et al., 2003; Moore et al., 2005). The contribution of *Campylobacter* spp. in pigs to human infection has been estimated at 10%, but this varies by country (Gillespie et al., 2002).

Campylobacter spp. are intestinal tract commensals in poultry, cattle, and swine; however, they can be associated with enteritis in calves and young pigs (Moore et al., 2005).

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Although pigs are carriers of *C. coli* and *C. jejuni*, *C. coli* are isolated more frequently than *C. jejuni* in this species (Harvey et al., 1999; Payot et al., 2004b). In addition, *C. coli* are also readily identified in environmental samples from swine production units (Leatherbarrow et al., 2004). Because campylobacteriosis is a zoonosis, AMR in *Campylobacter* spp. in food animals is a public health concern. Antimicrobial resistance among *Campylobacter* spp. that infect humans has increased in the last 15 years (Blaser and Engberg, 2008), and in general, *C. coli* are resistant to a larger number of antimicrobials than *C. jejuni* (Bywater et al., 2004). *Campylobacter* organisms that are resistant to tetracycline, ciprofloxacin, macrolides, chloramphenicol, aminoglycosides, ampicillin and other β-lactams, and trimethoprim-sulfamethoxazole have been isolated from animals including poultry and swine (Alfredson and Korolik, 2007). For human patients with campylobacteriosis, both azithromycin (Gilbert et al., 2007) and erythromycin (Bardon et al., 2008) may be used effectively when antimicrobial treatment is indicated. The therapeutic use of fluoroquinolones in human patients has been greatly reduced by the widespread development of fluoroquinolone-resistant *Campylobacter* strains worldwide (Taylor et al., 2008).

A review by (Andersson, 2003) revealed that continuous antimicrobial use exerts selective pressure that ultimately results in the emergence of resistant strains. In 2007, Alfredson and Korolik (Andersson, 2003) reviewed the results of several studies that indicated that fluoroquinolone resistance among *Campylobacter* spp. in humans increased following approval of a drug in the same class for use in food animals. However, cause and effect were not established. In addition, the presence of resistance

genes, whether derived from commensal bacteria or environmental sources, has been implicated in the increased incidence of resistance over time (Moore et al., 2005).

Mitigating AMR is necessary to prevent the emergence and dissemination of resistant strains and to ensure continued successful treatment of microbial infections in humans and animals. Antimicrobial use practices in agriculture may be an area in which intervention will reduce the prevalence of AMR determinants in the food chain. One such intervention is antimicrobial-free farming. Antimicrobial-free farming is defined as farming without the use of any antimicrobial drugs (Baker, 2006). However, reduction in antimicrobial drug use in food animals may lead to an increase in pathogen load (Casewell et al., 2003). The objective of the study reported here was to identify and compare the apparent prevalence of *Campylobacter* spp. and the apparent prevalence and patterns of AMR for fecal *Campylobacter* spp. isolated from pigs reared under antimicrobial-free and conventional production methods in the Midwestern United States.

2.2. Materials and Methods

2.2.1 Sample collection

This research was a part of a large study investigating the apparent prevalence of and risk factors associated with antimicrobial susceptibility patterns of *E. coli* and *Campylobacter* spp. isolated from finisher pigs on antimicrobial-free and conventionally managed farms in 8 states in the Midwestern United States. Results regarding AMR patterns in *E. coli* from the study have been reported (Bunner et al., 2007).

The present study included 95 farms in the Midwestern United States, including Iowa (n = 37), Illinois (15), Indiana (5), Michigan (21), Minnesota (8), Nebraska (6), Ohio (2), and Wisconsin (1). Sixty farms were managed under conventional swine farm practices, and 35 farms were considered antimicrobial-free facilities. The production systems that were classified as antimicrobial-free had not used antimicrobial drugs for a minimum of 1 year prior to enrollment in the study. Antimicrobial-free farms were selected from membership lists of 2 cooperatives; conventional farms were selected on the basis of close geographic proximity to the antimicrobial-free farms, or the number of slaughter pigs produced per year (Bunner et al., 2007). The total number of pigs marketed per year was used as a surrogate for herd size.

Samples of feces were collected from 15 pigs on each farm with the exception of 1 farm, where only 12 pigs were available for sample collection. Collection of feces from individual pigs on farms has been previously described (Bunner et al., 2007). Briefly, farms were visited once in 2002 or 2003, and samples were collected only from healthy pigs. Approximately 5 g of fresh fecal material/pig was collected and placed in a tube containing Cary-Blair transport medium (Medical Chemical Corp, Torrance, CA). The specimens were sent on ice to the National Food Safety and Toxicology Center, Michigan State University, and plated within 48 hours of collection.

2.2.2 Bacterial culture

Approximately 1 gram of fecal material/sample from the Cary-Blair transport medium was inoculated onto 1 blood agar plate (VMR, West Chester, PA). The inoculated plates were incubated for 48 hours at 42°C in a microaerophilic atmosphere

of 5% to 12% CO_2 and 5% to 15% O_2 (BBL Microbiology Systems, Cockeysville, MD). On each plate, 4 *Campylobacter*-like colonies were identified, if present, and plated on 1 of 4 quadrants on a blood agar plate and incubated. Colonies typical for *Campylobacter* spp. were further characterized by Gram stain results, microscopic appearance, and catalase and oxidase production in accordance with the standard methods at the National Food Safety and Toxicology Center, Michigan State University (Nachamkin, 1999). Isolates were identified as *Campylobacter* spp. if they were gram negative with a typical curved appearance microscopically, grew at 42°C under microaerophilic conditions, and were positive for catalase and oxidase. *Campylobacter jejuni* was further characterized by positive results of a hippurate hydrolysis test (Fitzgerald et al., 2008). Because some species of *Campylobacter* can be difficult to distinguish, a colony m-PCR assay was also used for isolate identification. *Campylobacter* isolates were frozen in 2% skimmed milk at –70°C in preparation for antimicrobial susceptibility testing and final identification by the use of the m-PCR procedure (Wang et al., 2002).

2.2.3 Differentiation of Campylobacter *spp.*

Pure cultures of *Campylobacter* spp. were thawed at room temperature (approx 20°C). Subsequently, *Campylobacter* spp. were differentiated by use of a colony m-PCR with slight modifications (Wang et al., 2002); the original PCR assay also identified *Campylobacter upsaliensis* and *Campylobacter fetus* subsp *fetus*. In brief, the m-PCR procedure identified the 23S rRNA from *Campylobacter* spp., the *hipO* gene (hippuricase) from *C. jejuni*, and the *glyA* gene (serine hydroxymethyltransferase) from *C. coli* and from *C. lari* by use of specific primer pairs (Appendix C) (Wang et al.,

2002). Primers and reagents were used in a 50-µL PCR system. The m-PCR assay mixture contained 1X *Taq*man buffer, 0.2mM deoxyribonucleotide triphosphate mix, 7.5mM $MgCl_2$, 0.5µM *C lari glyA* forward and reverse primers, 0.5µM *C jejuni hipO* forward and reverse primers, 1.0µM *C coli glyA* forward and reverse primers, 0.2µM *C jejuni* 23S rRNA forward and reverse primers, 0.05 U/ µL (2.5 units) *Taq* DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and approximately 10^6 whole bacterial cells. Amplification was achieved by use of a thermocycler (Bio-Rad Laboratories, Hercules, CA) with an initial denaturation step at 95°C for 6 minutes. An additional denaturation step at 95°C for 30 seconds followed by annealing at 59°C for 30 seconds and polymerization at 72°C for 30 seconds was repeated for 30 cycles. Final extension was carried out at 72°C for 7 minutes. Polymerase chain reaction products were separated on a 1.5% agarose gel at 90 V for 2.25 hours with ethydium bromide (0.5 µg/mL) added to the Tris, boric acid, EDTA buffer.

2.2.4 Assessment of AMR

Antimicrobial susceptibility testing was performed by use of commercially available gradient disk diffusion strips, Etest® (AB Biodisk, Piscataway, NJ), according to the manufacturer's instructions. Frozen bacterial isolates were thawed at room temperature, inoculated onto blood agar plates, and incubated at 42°C in a microaerophilic atmosphere (BBL Microbiology Systems, Cockeysville, MD) for a minimum of 48 hours. Typical colonies were selected and subcultured on plates containing trypticase soy agar with 5% sheep blood (VMR, West Chester, PA). These plates were incubated under microaerophilic conditions at 42°C for 48 hours. Colonies

from subculture were tested as described by Sato et al.(Sato et al., 2004a). Six antimicrobials were tested: azithromycin (0.016 to 256 μg/mL), erythromycin (0.016 to 256 μg/mL), ciprofloxacin (0.002 to 32 μg/mL), nalidixic acid (0.016 to 256 μg/mL), gentamicin (0.016 to 256 μg/mL), and tetracycline (0.016 to 256 μg/mL). The gradient disk diffusion strips provided 29 possible MIC values for each antimicrobial drug tested. For each antimicrobial drug, there were 15 possible $\log_2$ dilutions on a strip (eg, 0.016 through 256) and intermediate values between each $\log_2$ dilution. Intermediate values between $\log_2$ dilutions were rounded up to the higher $\log_2$ dilution during post study data management, as recommended by the manufacturer. *Campylobacter jejuni* (ATCC 3356022) and *E. coli* (ATCC 25922) were used as quality control strains. Resistance breakpoint (MacGowan and Wise, 2001) is defined as the MIC at which a bacterial isolate is considered resistant to a particular antimicrobial drug. Resistance breakpoints used by the National Antimicrobial Resistance Monitoring System were adopted (CDC, 2003). The resistance breakpoints were azithromycin (≥ 2 μg/mL), erythromycin (≥ 8 μg/mL), ciprofloxacin (≥ 4 μg/mL), nalidixic acid (≥ 32 μg/mL), gentamicin(≥ 16 μg/mL), and tetracycline (≥ 16 μg/mL).

2.2.5 Statistical analysis

Data regarding *Campylobacter* isolates, AMR, and farm management factors were compiled in a commercially available database software program (Microsoft Access, 2003, Microsoft Corp, Redmond, WA). Apparent prevalence is reported as a proportion with 95% exact CIs. Results of susceptibility testing are reported as MIC distributions and proportions of resistant and susceptible isolates according to the

Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). Statistical analysis was performed by use of a commercial software package (STATA, version 10.0, StataCorp, College Station, TX). Logistic regression analysis was used to test the associations between resistant isolates for each of the 6 antimicrobial agents and production method (i.e., conventional or antimicrobial-free farms) (Hosmer and Lemeshow, 2000). At the individual animal level, a population-averaged logistic regression model involving a generalized model framework with a logit link and binomial error distribution was used to determine the potential association between the proportion of resistance for each of the 6 antimicrobial agents and production method (Dohoo et al., 2003). A generalized estimating equation involving an exchangeable working correlation structure and semi-robust variance estimator was used to model within farm dependence (Hosmer and Lemeshow, 2000; Dohoo et al., 2003). Potential confounding effects by herd size and season were assessed for each antimicrobial agent. Season was defined as winter (January through March), spring (April through June), summer (July through September), and fall (October through December). Herd size was defined as the total number of finisher pigs marketed per year. Herd size was then dichotomized at a cutoff of 2,000 animals. The generalized Wald test was used to test significance (set at a value of $P < 0.05$) of independent variables in the models. Potential confounding variables were assessed by comparison of the differences in the regression coefficients with and without the presence of the potential confounder in the model. If there was a change of 20% or more, then adjusted measures of association were reported (Dohoo et al., 2003). Additionally, a possible dose-dependent relationship between the

number of years that antimicrobial drugs were not used on antimicrobial-free farms, and the level of resistance to the 6 antimicrobial drugs was investigated.

Pan-susceptible isolates were defined as those susceptible to all 6 antimicrobial drugs. Multidrug resistance was defined as resistance to 2 or more antimicrobial drugs. We assessed multidrug resistance using 2 approaches: specific and nonspecific MDR patterns. Nonspecific MDR was defined as resistance to any combination of ≥ 2 antimicrobials. Specific MDR was defined as resistance to a specific combination of ≥ 2 antimicrobials (e.g, azithromycin-erythromycin-tetracycline).

2.3. Results

2.3.1 Sampling

Fecal samples were collected from 1,422 pigs on antimicrobial-free (n = 35) and conventional (60) swine farms in the Midwestern United States. The number of years that antimicrobial drugs were not used on farms ranged from 1 to 14, with a median of 3 years. The mean number of pigs from farms that were considered antimicrobial-free production systems was 1,262 (range, 150 to 11,000; median, 800), whereas the mean number of pigs from conventional farms was 7,909 (range, 500 to 45,000; median, 4,800; $P < 0.001$). The proportions of antimicrobial-free and conventional farms evaluated in each season were as follows: winter, 11 of 35 (31%) and 22 of 60 (37%) farms, respectively; spring, 6 of 35 (17%) and 13 of 60 (22%) farms, respectively; summer, 8 of 35 (23%) and 9 of 60 (15%) farms, respectively; and fall, 10 of 35 (29%) and 16 of 60 (27%) farms, respectively.

2.3.2 Apparent prevalence of Campylobacter *spp.*

Culture results were positive for *Campylobacter* spp. for 1or more pigs on 90 of the 95 (94.7% [95% CI, 88.1% to 98.3%]) farms included in the study **(Table 2.1)**. Among the 35 antimicrobial-free farms, 33 (94.3% [95% CI, 80.8% to 99.3%]) had 1 or more *Campylobacter*-positive samples, and among the 60 conventional farms, 57 (95.0% [95% CI, 86.1% to 99.0%]) had 1 or more *Campylobacter*-positive samples. Across all farms, 512 fecal samples (36.0% [95% CI, 33.5% to 38.6%]) were positive for *Campylobacter* spp. Among antimicrobial-free farms, 190 of 522 (36.4% [95% CI, 32.3% to 40.7%]) samples were positive for *Campylobacter* spp. Among conventional farms, 322 of 900 (35.8% [95% CI, 32.6% to 39.0%]) isolates were *Campylobacter* spp. The herd-level and individual animal–level apparent prevalences were not significantly different between antimicrobial-free and conventional farms. In addition, herd size was not associated with apparent prevalence. The m-PCR assay was performed on 427 of the 512 isolates, and they were identified as *C. coli* (n = 426 [99.6%]) and *C. jejuni* (1 [0.4%]).

2.3.2 Antimicrobial susceptibility

Of the 512 *Campylobacter* spp. isolates, 464 (90.6%) were available for antimicrobial susceptibility testing; these isolates were obtained from 30 of 33 (90.9%) antimicrobial-free farms and 55 of 57 (96.5%) conventional farms that had ≥ 1 pig with positive culture results. Forty-eight (9.4%) samples across all samples were not recoverable after storage at –70°C; the unrecoverable samples included 16 of 190 (8.4%) samples collected from antimicrobial-free farms and 32 of 322 (9.9%) samples collected.

Table 2.1. Animal- and herd-level apparent prevalence of *Campylobacter* isolates from 1,422 fecal samples obtained from 35 antimicrobial-free and 60 conventional swine farms in the Midwestern United States.

Level	Farm type	No. of *Campylobacter* isolates/total No. of samples	Percentage of *Campylobacter* isolates (95% CI)	Median	Range	Odds ratio * (95% CI)	*P* value†
Animal	Conv	322/900	35.8 (32.6-39.0)	5	0-12	0.99 (0.91-1.09)	0.92
	ABF	190/522	36.4 (32.3-40.7)	6	0-13		
	Total	512/1,422	36.0 (33.5-38.6)	5	0-12		
Herd	Conv	57/60	95.0 (86.1-99.0)	NE	NE	1.15 (0.18-7.25)	0.88
	ABF	33/35	94.3 (80.8-99.3)	NE	NE		
	Total	90/95	94.7 (88.1-98.3)	NE	NE		

*Odds ratios were calculating by use of a population-averaged model (general estimating equations). †A value of $P \leq 0.05$ was considered significant.

ABF = Antimicrobial-free farm. Conv = Conventional farm. NE = Not estimable. CI = Confidence interval.

from conventional farms. Five (3/33 [9.1%] antimicrobial-free farms and 2/55 [3.6%] conventional) farms on which *Campylobacter* spp. were isolated from at least 1 pig had at least 1 sample that was not available for susceptibility testing.

At the farm level, the proportion of farms with 1 or more *Campylobacter* isolate resistant to azithromycin or to erythromycin was significantly ($P < 0.001$) higher for conventional farms, compared with antimicrobial-free farms **(Table 2.2).** The number of herds with at least 1 ciprofloxacin-or nalidixic acid–resistant isolate was higher for antimicrobial-free farms, compared with conventional farms. Conversely, the individual animal apparent prevalence of resistance to ciprofloxacin or nalidixic acid was greater on conventional farms **(Table 2.3)**.

The distributions of MICs were bimodal for azithromycin, erythromycin, ciprofloxacin, and nalidixic acid **(Table 2.4)**. The distribution of MICs for tetracycline was almost uniform across the various dilutions. Across farm type, significantly more *Campylobacter* isolates had a higher apparent prevalence of resistance to azithromycin, erythromycin, or tetracycline on conventional farms, compared with findings on antimicrobial-free farms ($P < 0.001$). For the macrolide antimicrobials erythromycin and azithromycin, the MIC_{50} value for each drug was 256 μg/mL for isolates obtained from conventional farms; for isolates obtained from antimicrobial-free farms, the MIC_{50} for azithromycin and erythromycin was 0.5 and 2 μg/mL, respectively. The MIC_{50} values for ciprofloxacin and nalidixic acid did not differ significantly between the two production systems, and none of the 464 isolates were resistant to gentamicin.

Table 2.2. Herd-level apparent prevalence of resistance to 6 antimicrobial agents in 464 *Campylobacter* isolates from 30 antimicrobial-free and 55 conventional swine farms in the Midwestern United States.

Antimicrobial drug	Farm type	No. of farms with ≥ 1 resistant isolate/total No. of farms	Percentage of farms with ≥ 1 resistant isolate (95% CI)	Odds ratio* (95% CI)	*P* value†
Azithromycin	Conv	52/55	94.5 (84.9-98.9)		
	ABF	14/30	46.7 (28.3-65.7)	0.05 (0.01-0.20)	< 0.001
Erythromycin	Conv	52/55	94.5 (84.9-98.9)		
	ABF	15/30	50.0 (31.3-68.7)	0.06 (0.02-0.23)	< 0.001
Ciprofloxacin	Conv	1/55	1.8 (0.05-9.7)		
	ABF	4/30	13.3 (3.8-30.7)	8.31 (0.88-78.09)	0.06
Nalidixic acid	Conv	3/55	5.5 (1.1-15.1)		
	ABF	4/30	13.3 (3.8-30.7)	2.67 (0.56-12.81)	0.22
Gentamicin	Conv	0/55	0 (0-6.5) ‡		
	ABF	0/30	0 (0-11.6) ‡	NE§	NE
Tetracycline	Conv	50/55	90.9 (80.0-97.0)		
	ABF	25/30	83.3 (65.3-94.4)	0.5 (0.13-1.89)	0.31

Conventional farms were the reference level.

‡One-sided 97.5% confidence interval.

§No farms had detectable *Campylobacter* isolates that were resistant to gentamicin and a measure of association was not estimable (NE).

See Table 2.1 for remainder of key.

Table 2.3. Prevalence of resistance of 6 antimicrobial agents and MIC (50% and 90%) of 464 *Campylobacter* isolates from 30 antimicrobial-free and 55 conventional swine farms.

Antimicrobial drug	Farm type	No. of resistant isolates/total No. of isolates	Percentage of resistant isolates (95% CI)	MIC_{50}	MIC_{90}	Odds Ratio (95% CI)	*P* value
Azithromycin	Conv	200/290	69.0 (63.3-74.3)	256	256		
	ABF	35/174	20.1 (14.4-26.8)	0.5	256	0.16 (0.07-0.38)**	<0.001
Erythromycin	Conv	198/290	68.3 (62.6-73.6)	256	256		
	ABF	37/174	21.3 (15.4-28.1)	2	256	0.16 (0.07 -0.37)**	<0.001
Ciprofloxacin	Conv	11/290	3.8 (1.9-6.7)	0.125	0.25		
	ABF	6/174	3.4 (1.3-7.4)	0.125	0.25	0.91 (0.09-8.74)[&]	0.93
Nalidixic Acid	Conv	13/290	4.5 (2.4-7.5)	4	8		
	ABF	6/174	3.4 (1.3-7.4)	4	8	0.94 (0.16 – 5.63)	0.94
Gentamicin	Conv	0/290	0 (0-1.3)*	1	1		
	ABF	0/174	0 (0-2.1)*	1	1	NE[†]	NE[†]
Tetracycline	Conv	216/290	74.5 (69.1-79.4)	64	256		
	ABF	85/174	48.8 (41.2-56.5)	8	256	0.17 (0.06 – 0.50)**	<0.001

Conventional farms were the reference level. Odds ratios were adjusted for confounding by herd size.

*One sided 97.5% CI

See Table 2.1 for remainder of key.

Table 2.4. Results of antimicrobial susceptibility testing of 464 *Campylobacter* isolates obtained from fecal samples from finisher pigs on 35 antimicrobial-free and 60 conventional swine farms in the Midwestern United States.

Antimicrobial drug	MIC (µg/mL)	ABF (No. [%] of isolates)	Conv (No. [%] of isolates)	Antimicrobial drug	MIC (µg/mL)	ABF (No. [%] of isolates)	Conv (No. [%] of isolates)
Azithromycin	≤ 0.016	0 (0.0)	0 (0.0)	Nalidixic Acid	≤ 0.016	0 (0.0)	0 (0.0)
	0.03	0 (0.0)	0 (0.0)		0.03	0 (0.0)	0 (0.0)
	0.064	1 (0.6)	4 (1.4)		0.064	0 (0.0)	0 (0.0)
	0.125	34 (19.5)	7 (2.4)		0.125	0 (0.0)	0 (0.0)
	0.25	39 (22.4)	39 (13.5)		0.25	0 (0.0)	0 (0.0)
	0.5	38 (21.8)	31 (10.7)		0.5	0 (0.0)	0 (0.0)
	1	27 (15.5)	9 (3.1)		1	13 (7.5)	10 (3.5)
Breakpoint, ≥2 µg/mL	2	2 (1.2)	2 (0.7)		2	71 (40.8)	123 (42.4)
	4	0 (0.0)	0 (0.0)		4	71 (40.8)	115 (39.7)
	8	0 (0.0)	0 (0.0)		8	12 (6.9)	27 (9.3)
	16	0 (0.0)	0 (0.0)		16	1 (0.6)	2 (0.7)
	32	0 (0.0)	0 (0.0)	Breakpoint, ≥ 32 µg/mL	32	0 (0.0)	2 (0.7)
	64	0 (0.0)	0 (0.0)		64	0 (0.0)	0 (0.0)
	128	0 (0.0)	0 (0.0)		128	2 (1.2)	0 (0.0)
	≥256	33 (19.0)	198 (68.3)		≥256	4 (2.3)	11 (3.8)

Table 2.4 (continued)

Antimicrobial drug	MIC (µg/mL)	ABF (No. [%] of isolates)	Conv (No. [%] of isolates)	Antimicrobial drug	MIC (µg/mL)	ABF (No. [%] of isolates)	Conv (No. [%] of isolates)
Erythromycin	≤ 0.016	0 (0.0)	0 (0.0)	Gentamicin	≤ 0.016	0 (0.0)	0 (0.0)
	0.03	0 (0.0)	0 (0.0)		0.03	0 (0.0)	0 (0.0)
	0.064	0 (0.0)	0 (0.0)		0.064	0 (0.0)	0 (0.0)
	0.125	0 (0.0)	0 (0.0)		0.125	0 (0.0)	0 (0.0)
	0.25	2 (1.2)	1 (0.3)		0.25	1 (0.6)	8 (2.8)
	0.5	8 (4.6)	7 (2.4)		0.5	76 (43.7)	118 (40.7)
	1	40 (22.9)	21 (7.2)		1	153 (52.3)	153 (52.8)
	2	57 (32.8)	41 (14.1)		2	5 (2.9)	10 (3.5)
	4	30 (17.2)	22 (7.6)		4	1 (0.6)	0 (0.0)
Breakpoint, ≥ 8 µg/mL	8	4 (2.3)	3 (1.0)		8	0 (0.0)	1 (0.3)
	16	0 (0.0)	0 (0.0)	Breakpoint, ≥ 16 µg/mL	16	0 (0.0)	0 (0.0)
	32	0 (0.0)	1 (0.3)		32	0 (0.0)	0 (0.0)
	64	1 (0.6)	1 (0.3)		64	0 (0.0)	0 (0.0)
	128	0 (0.0)	0 (0.0)		128	0 (0.0)	0 (0.0)
	≥256	32 (18.4)	193 (66.6)		≥256	0 (0.0)	0 (0.0)

Table 2.4 (continued)

Antimicrobial drug	MIC (µg/mL)	ABF (No. [%] of isolates)	Conv (No. [%] of isolates)
Ciprofloxacin	≤ 0.016	1 (0.6*)	3 (1*)
	0.03	5 (2.9)	25 (8.6)
	0.064	45 (25.9)	82 (28.3)
	0.125	77 (44.3)	111 (38.3)
	0.25	33 (19.0)	50 (17.2)
	0.5	7 (4.0)	7 (2.4)
	1	0 (0.0)	1 (0.3)
	2	0 (0.0)	0 (0.0)
Breakpoint, ≥ 4 µg/mL	4	0 (0.0)	0 (0.0)
	8	0 (0.0)	0 (0.0)
	16	0 (0.0)	0 (0.0)
	32	6 (3.5)	11 (3.8)
Tetracycline	≤ 0.016	0 (0.0)	0 (0.0)
	0.03	1 (0.6)	0 (0.0)
	0.064	2 (1.2)	1 (0.3)
	0.125	12 (6.9)	3 (1)
	0.25	18 (10.3)	2 (0.7)
	0.5	26 (14.9)	7 (2.4)
	1	8 (4.6)	7 (2.4)
	2	6 (3.5)	16 (5.5)
	4	7 (4.0)	19 (6.6)
	8	9 (5.2)	19 (6.6)
Breakpoint, ≥ 16 µg/mL	16	8 (4.6)	21 (7.2)
	32	19 (10.9)	40 (13.8)
	64	15 (8.6)	29 (10.0)
	128	13 (7.5)	27 (9.3)
	≥256	30 (17.2)	99 (34.1)

ABF=Antimicrobial-free farms. Conv=Conventional farms.
*Dilution's for ciprofloxacin ranged from 0.02-32µg/mL; all samples tested at dilutions ≤ 0.016 µg/mL were combined.

Inclusion of season as a potential confounder in the statistical model did not change the association between production system and AMR. Inclusion of herd size in the model changed the overall effect of production method (conventional and antimicrobial free) on AMR prevalence by more than 20%; therefore, herd size was considered a confounder. To account for the confounding effect of herd size on the model, herd size was forced into each model for all 6 antimicrobial drugs. In addition, herd size was added to the models investigating nonspecific and specific resistance patterns. However, there was no significant interaction between herd size and production system type. In the present study, nondifferential misclassification was unlikely since the culture and MIC methods were equivalent for antimicrobial-free and conventional farms. In either case, the effects of nondifferential misclassification would likely bias the estimates of association in this study toward a null (a more conservative *P* value).

As the number of years that an antimicrobial-free production scheme was implemented on a farm increased, there was a significant ($P = 0.002$ for the first 2 years then $P < 0.001$ for years 3 to 15) and consistent decrease by year in the proportion of isolates that were resistant to azithromycin or erythromycin **(Table 2.5)**. Resistance to tetracycline did not decrease consistently as the duration of antimicrobial-free production increased, but after 3 years, the number of resistant strains was significantly ($P < 0.001$) less, compared with the number of resistant strains on conventional farms. On antimicrobial-free farms on which antimicrobial drugs had not been used for 6 or more years, the apparent prevalence of resistance to tetracycline was 40% less than that of conventional farms; the apparent prevalences of resistance to azithromycin and

erythromycin were each 83% less than that of conventional farms.

Ten specific resistance patterns to 2 or more of 5 antimicrobial drugs were identified (none of the isolates were resistant to gentamicin; **Table 2.6**). The most common pattern was resistance to azithromycin, erythromycin, and tetracycline, which was significantly ($P < 0.001$) higher on conventional farms than on antimicrobial-free farms. The proportion of pan-susceptible isolates was higher on antimicrobial-free farms (42.5% [95% CI, 35.1% to 49.0%]), compared with the proportion on conventional farms (7.9% [95% CI, 4.8% to 11.1%]; **Figure 2.1**). Across both production systems, one isolate was resistant to 4 (azithromycin, erythromycin, nalidixic acid, and tetracycline) antimicrobial drugs, and one isolate was resistant to 5 (azithromycin, erythromycin, nalidixic acid, tetracycline, and ciprofloxacin) antimicrobial drugs.

2.4. Discussion

In the present cross-sectional study, *Campylobacter* spp. were isolated from approximately a third of samples collected on both conventional and antimicrobial-free swine farms. This is within the previously reported range of *Campylobacter* spp. apparent prevalence among finishing pigs (16% to 100%) (Harvey et al., 1999; Payot et al., 2004b; Gebreyes et al., 2005) including findings of one study (Thakur and Gebreyes, 2005) that compared prevalence in antimicrobial-free and conventional production systems (53% and 55.8%, respectively). A similar study (Sato et al., 2004a) in cows also did not identify a significant difference in prevalence between the two production systems. Often, shedding of pathogens is greater in larger herds (Fossler et al., 2005);

however, in our study, *Campylobacter* spp. apparent prevalence was not associated with herd size. In addition, 95% of all farms had at least one *Campylobacter*-positive pig, which suggests that *Campylobacter* spp. are widespread. The results of the present study further emphasize that pigs are common reservoirs for *Campylobacter* spp., regardless of production system and herd size.

At the farm level, resistance of *Campylobacter* spp. to azithromycin or erythromycin for one or more individual pigs/farm was detected on most of the conventional farms, yet resistance to each of these macrolides was detected on approximately half as many antimicrobial-free farms. The lack of recent exposure to macrolides may have contributed to the lower number of antimicrobial-free farms with resistance to macrolides because of a reduction in selective pressure. Resistance to macrolides may confer a fitness cost (a decrease in the ability of a bacterium to compete with other bacteria in the environment) that would cause bacteria that acquire additional resistance genes to become less fit (Andersson, 2003). Tetracycline resistance was evident on almost all conventional and antimicrobial-free farms (7% difference). This may result from mutations that confer resistance without reducing the fitness of the bacteria, or from environmental persistence of plasmid-mediated resistance genes associated with *Campylobacter* spp. resistance to tetracycline (Andersson, 2003; Jindal et al., 2006). Resistance to ciprofloxacin or nalidixic acid was rare and was evident on

Table 2.5 Effect of years of antimicrobial-free production on prevalence of antimicrobial resistance among *Campylobacter* isolates from 30 antimicrobial-free and 55 conventional Midwestern swine farms. Three antimicrobials (ciprofloxacin, nalidixic acid, and gentamicin) did not have enough observations to calculate odds ratios.

Antimicrobial drug	Farm type and years antimicrobial-free	No. of resistant isolates / total No. of isolates	No. of farms	Percentage of resistant isolates (95% CI)	Odds ratio (95% CI)	*P* value
Azithromycin	Conv	200/290	55	69.0 (63.6-74.3)		
	ABF (1-2 y)	11/29	7	37.9 (20.7-57.7)	0.23 (0.09-0.57)	0.002
	ABF (3 y)	12/55	9	21.8 (11.8-35.0)	0.09 (0.03-0.28)	< 0.001
	ABF (4-5 y)	7/47	8	14.9 (6.2-28.3)	0.06 (0.02-0.22)	< 0.001
	ABF (≥ 6 y)	5/43	6	11.6 (3.9-25.1)	0.04 (0.01-0.17)	< 0.001
Erythromycin	Conv	198/290	55	68.3 (62.7-73.6)		
	ABF (1-2 y)	11/29	7	37.9 (20.7-57.7)	0.25 (0.10-0.61)	0.002
	ABF (3 y)	12/55	9	21.8 (11.8-35.0)	0.11(0.04-0.31)	< 0.001
	ABF (4-5 y)	8/47	8	17.0 (7.6-30.8)	0.07 (0.02-0.23)	< 0.001
	ABF (≥ 6 y)	6/43	6	14.0 (5.3-27.9)	0.06 (0.02-0.21)	< 0.001
Tetracycline	Conv	216/290	55	74.5 (69.1-79.4)		
	ABF (1-2 y)	17/29	7	58.6 (30.9-76.5)	0.47 (0.16-1.36)	0.164
	ABF (3 y)	34/55	9	61.8 (47.7-74.6)	0.58 (0.22-1.53)	0.272
	ABF (4-5 y)	15/47	8	31.9 (19.1-47.1)	0.17 (0.07-0.39)	< 0.001
	ABF (≥ 6 y)	19/43	6	44.2 (29.1-60.1)	0.24 (0.12-0.52)	< 0.001

Odds ratios were adjusted for confounding by herd size.
See Table 2.1 for remainder of key.

Table 2.6 Specific patterns of resistance among 464 *Campylobacter* isolates recovered from finisher pigs on 35 antimicrobial-free and 60 conventional Midwestern swine farms.

Antimicrobial drug combination	Farm Type	No. of resistant isolates/total No. of isolates	Percentage of resistant isolates (95% CI)	Odds ratio (95% CI)	*P* value
None	Conv	23/290	7.9 (5.1-11.7)		
	ABF	74/174	42.5 (35.1-50.2)	10.60 (2.15-52.12)	0.004
Azithromycin-erythromycin	Conv	37/290	12.8 (9.1-17.2)		
	ABF	12/174	6.9 (3.6-11.7)	1.07(0.37-3.09)	0.9
Ciprofloxacin-nalidixic acid	Conv	10/290	3.4 (1.7-6.2)		
	ABF	2/174	1.1 (-0.1-4.1)	0.81 (0.16-4.06)	0.8
Azithromycin-erythromycin-tetracycline	Conv	157/290	54.1 (48.2-60.0)		
	ABF	21/174	12.1 (7.6-17.8)	0.13(0.05-0.32)	< 0.001

Conventional farms were the reference level. Odds ratios were adjusted for confounding by herd size.
See Table 2.1 for key.

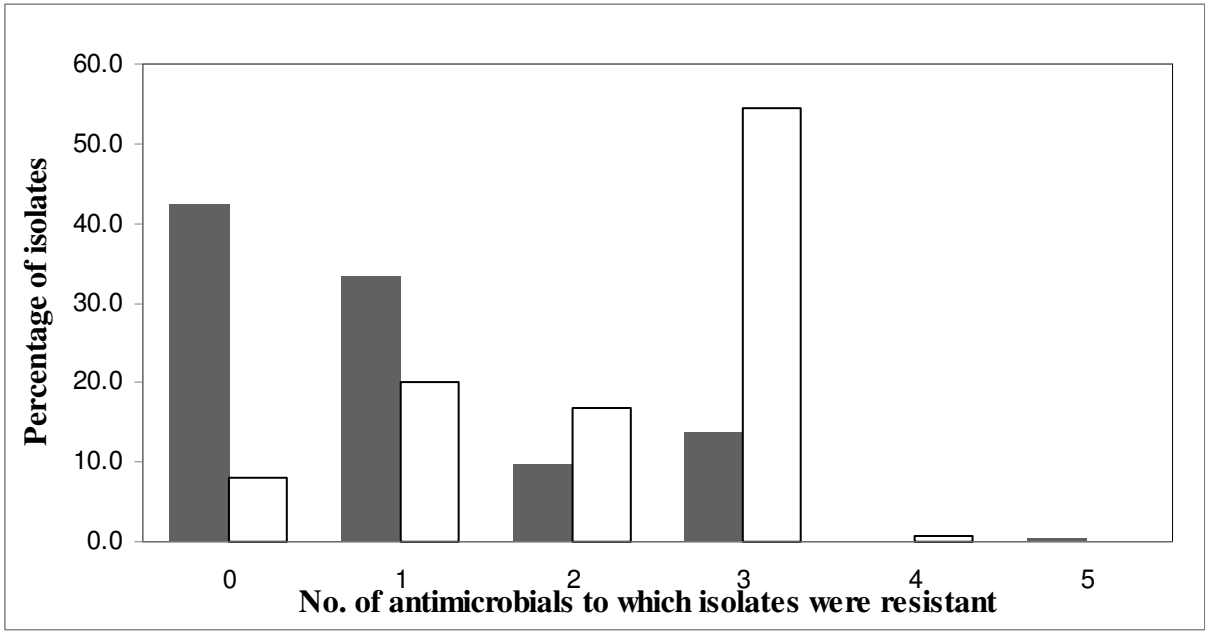


Figure 2.1. Nonspecific MDR among 464 *Campylobacter* isolates from 35 antimicrobial-free (black bars) and 60 conventional (white bars) swine farms.

more antimicrobial-free than conventional farms; this may have resulted from unidentified mechanisms, possibly including adaptation of resistant strains or the presence of efflux pumps (Luo et al., 2005). These efflux pumps limit access of antimicrobial drugs to their targets by actively pumping out these molecules (Kohler et al., 1999).

At the animal level, the highest apparent prevalences of AMR were to erythromycin, azithromycin, and tetracycline. This finding was similar to the results of other studies (Payot et al., 2004b; Thakur and Gebreyes, 2005). Resistances to the macrolide antimicrobial drugs (azithromycin and erythromycin) were approximately 70% higher on conventional than antimicrobial-free farms. In addition, conventional farms had a higher proportion of isolates resistant to high concentrations of macrolides (MIC $\geq$ 256 μg/mL). One explanation for the high prevalence of macrolide resistance may be the use of tylosin, a macrolide, which is approved for use for growth promotion and therapeutic purposes in swine (Lin et al., 2007; Zhang and Plummer, 2008). Antimicrobial-free farms that lack exposure to macrolides might be expected to eliminate the high concentration–resistant strains first.

The high level of erythromycin and azithromycin resistance on conventional farms is of concern because erythromycin and azithromycin are currently the most common antimicrobial treatments for *Campylobacter* infections in humans (Guerrant et al., 2001; Gilbert et al., 2007). Erythromycin and azithromycin resistances result from a chromosomal mutation of the ribosome 23S rRNA genes or genes encoding ribosomal proteins L4 and L22, not from horizontally acquired genes from other bacteria (Engberg

et al., 2001; Zhang and Plummer, 2008). Erythromycin and azithromycin resistances are rare in *C. jejuni* but common among *C. coli* strains, particularly among isolates from pigs and pig offal (Moore et al., 2006). The higher prevalence of resistance to erythromycin in pigs has not been fully explained; however, Engberg et al (Engberg et al., 2001) suggest this may be due to a generally higher frequency of mutations conferring resistance among *C. coli*, or due to greater selective pressure resulting from prior use of antimicrobial agents. In addition, *C. coli* also has an efflux pump system that contributes to acquired resistance to macrolides (Gibreel et al., 2007).

The antimicrobial drug with the highest apparent prevalence of resistance on antimicrobial-free and conventional farms was tetracycline (49% and 75%, respectively). The high apparent prevalence of tetracycline resistance is most likely due to the presence of the genetic determinant *tet(O)* on transferable plasmids that prevent tetracycline from binding to the ribosome, as well as the presence of efflux pumps (Pumbwe and Piddock, 2002; Dasti et al., 2007). The most common mechanism involves the plasmid encoded *tet(O)* gene, which produces a ribosomal protection protein that confers resistance by preventing tetracycline from binding to the ribosome (Moore et al., 2005; Dasti et al., 2007). *Tet(O)* is commonly found in a variety of bacteria in farming environments (Jindal et al., 2006) and in pig samples, regardless of prior antimicrobial usage (Aminov et al., 2001). Comparison of *C. jejuni* and *C. coli* isolates derived from humans (Dasti et al., 2007) established that all *tet(O)* genes among *C. coli* were chromosomally related, rather than carried by plasmids as is the case for *C. jejuni*. If *tet(O)* genes are chromosomally related among *C. coli* derived from swine, then this is an important

distinction that should be further investigated in food animals, because *C. coli* is the predominant subspecies in swine. Also if *tet(O)* genes are chromosomally related in *C. coli* derived from swine, this may then explain epidemiological differences between swine and other food animals. In addition to transferable plasmids, the multidrug efflux pump CmeABC contributes to intrinsic and acquired resistance (Lin et al., 2002; Gibreel et al., 2007). The multiple and complex resistance mechanisms of tetracycline are a likely explanation for the high proportion of resistant isolates and the broad characteristic MIC values observed for tetracycline.

The present and previous studies (Thakur and Gebreyes, 2005; Price et al., 2007) have identified resistance of *Campylobacter* spp. to ciprofloxacin in both conventional and antimicrobial-free farming systems in North America. The presence of fluoroquinolone resistance in both production systems is of particular concern because this class of drugs was not approved for use in swine production at the time of our study (van den Bogaard and Stobberingh, 2000). In addition, in a study (Luo et al., 2003) of chickens, fluoroquinolone-resistant *Campylobacter* spp. colonized and persisted in chickens as efficiently as susceptible strains in the absence of fluoroquinolone antimicrobials. The *gyr*A and *par*C genes are responsible for production of DNA gyrase and toposiomerase IV, the proteins that are targets for fluoroquinolones. *Campylobacter* spp. do not produce toposiomerase; hence a single mutation in *gyrA* gene can cause a high level of resistance to fluoroquinolones ($\geq$ 32 μg/mL) (Luo et al., 2003; Ge et al., 2005). Furthermore, the most frequently reported mechanism of resistance to fluoroquinolones is the target mutation of the *gyr*A gene; at least 4 unique point

mutations in the *gyr*A gene of the fluoroquinolone-resistant mutants, resulting in high and intermediate levels of resistance of *Campylobacter* spp. to the fluoroquinolones, have been reported (Payot et al., 2006; Zhang and Plummer, 2008). In addition, the CmeABC efflux pump is associated with fluoroquinolone resistance in *Campylobacter* spp. (Luo et al., 2003; Fabrega et al., 2008). In the present study, isolates resistant to ciprofloxacin and nalidixic acid were distributed between 4 antimicrobial-free farms and one conventional farm that, combined, had 11 pigs with ciprofloxacin-resistant *Campylobacter* spp. Hence, ciprofloxacin resistance among *Campylobacter* spp. appears to be present only on certain farms. Furthermore, it is unknown how long ciprofloxacin-resistant *Campylobacter* organisms have been present on the antimicrobial-free farms in our study. In poultry, resistance of *Campylobacter* spp. to fluoroquinolones persisted for at least 4 years after cessation of antimicrobial usage (Pedersen and Wedderkopp, 2003). In our study, the data are insufficient to make inferences regarding exposure and resistance. Further studies should concentrate on examination of risk factors that might be expected to promote the presence or persistence of ciprofloxacin resistance on swine farms.

An apparent dose-response effect was observed for the duration of antimicrobial-free production (1 to 14 years). The gradual wane in azithromycin and erythromycin resistances over time was expected because their resistance mechanisms have a chromosomal linkage and would only be transmitted vertically. Following mutation, there is often a fitness deficit of the bacteria conferred by resistance (Zhang et al., 2006); therefore, susceptible strains may become more predominant over time in the absence of

antimicrobial pressure. However, in our study, tetracycline resistance had a threshold decline after 3 years, or in other words, tetracycline resistance did not decline until a farm was antimicrobial free for 3 or more years. The large variety of mechanisms of tetracycline resistance among *Campylobacter* spp. isolates may explain why there was only a 40% decrease in tetracycline resistance on farms that were antimicrobial free for ≥ 6 years, compared with findings on conventional farms; in contrast, an 80% decrease in erythromycin resistance and an 83% decrease in azithromycin resistance was detected between those farm types. Ciprofloxacin and nalidixic acid did not have a sufficient number of resistant isolates to detect a pattern.

Considering the predominant mechanism of resistance for each antimicrobial tested, the resistance patterns detected in the present study were expected. However, we compared 2 production methods at a single point in time, so the assumption was made that antimicrobial-free farms had AMR prevalences similar to those on conventional farms prior to the cessation of antimicrobial use. Although caution is needed in making inferences about a true dose effect, these patterns can serve to generate hypotheses regarding why resistance to some antimicrobials but not to others appears to change over time.

Multidrug resistance was common in the present study. In 3 other studies, (Payot et al., 2004b; Gebreyes et al., 2005; Thakur and Gebreyes, 2005) the most common MDR in *C. coli* in pigs was the combination of erythromycin, nalidixic acid, and tetracycline. In our study, this combination was also present on conventional farms (0.34% of total MDR combinations). Two isolates were resistant to 4 or 5 antimicrobial agents, including erythromycin, ciprofloxacin, and tetracycline, which may be used to treat human infections. Multidrug resistance in *Campylobacter* spp. is most commonly due to the presence of multidrug efflux pumps, which contribute to the intrinsic resistance of *Campylobacter* spp. to a broad range of structurally unrelated antimicrobial agents (Lin et al., 2002; Payot et al., 2004b; Moore et al., 2006). As previously noted, resistances of *Campylobacter* spp. to fluoroquinolones and macrolides result from mutations of the *gyr*A or 23S rRNA gene, respectively.

In a recent review, Payot et al (Payot et al., 2006) concluded that the CmeABC efflux system works synergistically with these mutations to confer high-level resistance to fluoroquinolones and macrolides. However, in the present study, only 2 of 17 (11.8%) *Campylobacter* isolates that were resistant to fluoroquinolones were also resistant to erythromycin or azithromycin. At present, the mechanisms of MDR in *Campylobacter* spp. are still incompletely understood; however, it appears that the role of efflux pumps should be a focus of further research in this area.

Campylobacteriosis in humans is primarily associated with consumption of food animal products (Jacobs-Reitsma et al., 2008). Intuitively, removal of antimicrobials from a production system should decrease AMR. In the study reported here, decreased AMR to erythromycin, azithromycin, and tetracycline was observed on antimicrobial-free farms. However, one issue with cross-sectional studies is that the rate of decrease in resistance cannot be directly quantified. In our study, the assumption was made that prior to cessation of antimicrobial use on antimicrobial-free farms, the proportions of *Campylobacter* spp. resistant to the antimicrobials tested were the same as the proportions on conventional farms. The cessation of antimicrobial use is a major production change, the benefits of which have yet to be fully examined. The changes in risk factors associated with this production change may inherently affect the outcome.

For example, antimicrobial-free farms are typically small and may use different management procedures that may affect risk factors differently than on conventional farms. Results of the present study suggest that AMR is greater on conventional farms; long-term prospective studies are indicated to examine whether these differences persist, and to compare specific risk factors in conventional farming environments with antimicrobial-free farms that lack antimicrobial selection pressure.

3. USING DISCRETE TIME SURVIVAL ANALYSIS TO MODEL THE DISTRIBUTION OF MINIMUM INHIBITORY CONCENTRATIONS OF ANTIMICROBIAL DRUGS IN *Campylobacter* SPP. AND *Escherichia coli* ISOLATED FROM FECES OF ANTIMICROBIAL-FREE AND CONVENTIONALLY RAISED SWINE

3.1. Introduction

Although contentious, it is argued that antimicrobial-resistant bacteria from food animals is a major public health concern (Chiller et al., 2004; Jensen et al., 2004; Karp and Engberg, 2004; Phillips et al., 2004). These arguments have led to suggestions to reduce the number and uses of antimicrobial drugs in food animal medicine (Wierup, 2001; Emborg et al., 2003; Grave et al., 2006; FDA, 2000, 2003). The discussions about antimicrobial drug use and its potential negative impact on public health have also prompted some producers and producer groups to voluntarily eliminate antimicrobial drug use in their food animal production systems. The idea behind ceasing antimicrobial drug use is that it will reduce the levels of antimicrobial resistance in bacteria isolated from animals reared without antimicrobial drug use as compared to animals raised in a production system where drugs are used for prevention and therapeutic uses.

A number of cross-sectional or have been used to compare the proportions of antimicrobial resistance among *Campylobacter* spp., *Salmonella*, and *Escherichia coli* isolated from food animal populations reared with and without antimicrobial drug use (Mathew et al., 2001; Englen et al., 2005; Gebreyes et al., 2005; Halbert et al., 2006;

Ray et al., 2006; Bunner et al., 2007; Rollo et al., 2010). In most of these studies, the cessation of antimicrobial drug use reduced the proportion of bacteria resistant to some antimicrobial drugs while it seemed to have little effect on resistance to other antimicrobial drugs.

Using microbiological breakpoints to dichotomize bacteria into susceptible and resistant categories may limit researchers' ability to compare susceptibility among bacteria isolated from animals reared under different production systems; particularly if none or very few of the bacteria have MIC values above the breakpoint (i.e., are classified as resistant). An alternative to comparing the proportions in two or more populations is to compare the distribution of MIC values in these populations. However, comparison of MIC distributions is inherently more complicated than comparing two proportions. First, MIC data are grouped in discrete categories (i.e., dilutions), and the distribution of MIC values may be right- or left-censored. Furthermore, the number of categories varies depending on the type of susceptibility test used. Using Etest®, there typically are 15 categories; however, for other methods such as microbroth dilution, there may be varying but smaller numbers (typically, two to six categories), increasing the probability of censored data. Some researchers have used survival analysis to model MIC distribution data (Ray et al., 2006; Stegeman et al., 2006; Pol and Ruegg, 2007). Survival analysis data consists of time to event measurements (i.e., event: yes or no; and, time under observation). In this case, the highest dilution or concentration of antimicrobial at which growth is exhibited for the particular isolate is the yes/no outcome and the intervals from the lowest dilution up to the MIC value recorded for an

isolate are the 'time' to the event. Because of the potential for a large number of ties and censored data, discrete-time survival analysis model (DTSA) is particularly well-suited to modeling MIC data.

Using microbiological breakpoints to dichotomize bacteria into susceptible and resistant categories also makes assumptions as to what the appropriate breakpoint is. An antibiotic breakpoint is an MIC that divides bacteria isolates into categories: susceptible, intermediate, and resistant. The definition of susceptible is the antimicrobial drug treatment is associated with a high likelihood of therapeutic success. Intermediate is associated with an uncertain therapeutic success, and resistant is associated with a higher than expected likelihood of therapeutic failure (Kahlmeter et al., 2003). The minimum inhibitory concentration determined *in vitro* is associated with the concentration of an antimicrobial that would effectively inhibit or kill the bacteria within the host at a species' anatomic level (Lorian, 2005). Clinically, breakpoints divide a population of bacterial isolates into those that are likely to be susceptible to treatment and those that are likely to be resistant to treatment. From a bacterial perspective, it would be useful to define the dilution in the distribution at which the bacterial population is divided into those that have resistance genes that cause them to differ from the wild-type bacteria (MacGowan and Wise, 2005). This has been referred to as the 'epidemiologic' breakpoint, although these breakpoints have been defined for only a few bacterial species (Lorian, 2005). If breakpoints are too conservative, borderline susceptible bacteria may be considered fully susceptible (Dalhoff et al., 2009). Several agencies including the European committee on antimicrobial susceptibility testing, (EUCAST), in

Europe or CLSI in North America determine breakpoints by either probabilistic methods or by deterministic methods. The new probabilistic approach may be favored because the pharmacokinetic and microbiologic variables are determined in addition to data from a large number of MIC/drug exposure scenarios (Dalhoff et al., 2009); however, this method is not currently utilized by CLSI. By using the entire MIC distribution as an outcome measure as well as the drug exposure distribution, the assumptions that are inherent in using a breakpoint are conveniently eliminated and comparisons across the years are facilitated.

The frequency distribution of resistance MICs among a group of bacterial isolates will not necessarily be normal. The distribution can be right or left censored, and often there will be a spike in the highest MIC category because some isolates will continue to grow at the highest dilution of the test; however, these will be grouped into the highest dilution despite being 'right-censored' (indeterminate MIC). In addition, the distribution is not truly continuous because the values are grouped into discrete intervals (i.e., dilutions).

The objective of this study was to introduce a statistical model that accounts for censoring and uses discrete time series (Ananth and Kleinbaum, 1997) to compare MIC distributions of *E. coli* and *Campylobacter* spp. isolated from antimicrobial-free and conventional swine farms in the Midwest.

3.2. Materials and Methods

3.2.1 Study design, collection and testing of samples

The study included data collected from 95 swine farms in the Midwestern United States. Sixty farms used conventional production methods and 35 farms were managed as antimicrobial-free farms. The antimicrobial-free farms, by definition, had not used antimicrobials for at least one year prior to being included in the study. Descriptive results regarding AMR levels and patterns in *Campylobacter* and in *E. coli* from this study have been reported elsewhere (Bunner et al., 2007; Rollo et al., 2010).

While visiting the 95 enrolled farms, fecal samples were collected from 15 pigs that were in the final stages of production, with the exception of one farm, where only 12 pigs were sampled. Sampling method, bacterial culture methods, and identification of *Campylobacter* spp. have been described previously (Bunner et al., 2007; Rollo et al., 2010). Likewise, antimicrobial susceptibility testing has also been described previously (Bunner et al., 2007; Rollo et al., 2010). Briefly, antimicrobial susceptibility of *Campylobacter* spp. was performed using a gradient disk diffusion strip, Etest® (AB Biodisk, Piscataway, NJ). Six antimicrobial drugs were used for *Campylobacter* spp. isolates; however, only data on azithromycin (0.016-256 ug/mL), gentamicin (0.016-256 ug/mL), and tetracycline (0.016-256 ug/mL) were used in this study. For *E. coli*, a microbroth dilution method (Trek Diagnostics, Westlake, OH) was used to determine the MICs to 14 antimicrobials for each isolate. The broth microdilution method has been described by Bunner (2007). For the study described here, three antimicrobials were examined: ampicillin (1-32 ug/mL), chloramphenicol (2-32 ug/mL), and gentamicin

(0.25-16 ug/mL). A subset of antimicrobials for each bacterial species was chosen for this project because they each have a unique shape for their respective MIC distributions.

The MIC value, defined as the lowest antimicrobial concentration that inhibited bacterial growth, was reported for each isolate. For isolates that did not exhibit growth inhibition at even the highest antimicrobial concentration – for each respective antimicrobial drug and susceptibility test (microbroth dilution and gradient diffusion test), the highest concentration was reported as the MIC. This is one of the discrepancies of using MIC distribution data without considering censoring; all of the isolates whose growth was not inhibited are grouped into this category.

3.2.2 Description of data and descriptive analysis

Data regarding *Campylobacter* spp. isolates, *E. coli* isolates, minimum inhibitory concentrations, and farm management factors were compiled in a commercially available database software program (Microsoft Access, 2003, Microsoft Corp, Redmond, WA). Statistical analysis was performed by use of commercial software package (STATA, version 10.0, Statacorp, College Station, TX).

MIC data were converted logarithmically using a log base 2 transformation. Discrete-time-series survival analysis was used to examine differences in MIC distributions for all six antimicrobial-bacterial combinations and between the two swine production methods. Kaplan-Meier survival curves were first produced to visually compare the MIC distributions (STATA, version 10.0, StataCorp, College Station, TX). For DTSA and Kaplan-Meier, Etest® values at or greater than 256 µg/ml (for

azithromycin, gentamicin and tetracycline) were treated as right-censored. Similarly, microbroth dilution values greater than 32 µg/ml for ampicillin, 32 µg/ml for chloramphenicol, and 16 µg/ml for tetracycline for *E. coli* were considered right-censored.

The Kaplan-Meier estimator of the survival function was calculated for each bacteria-antimicrobial combination. This estimator used all isolates including censored ones to calculate a cumulative survival probability at each observed interval. Each isolate was included in the denominator or as 'at risk' isolates for inhibition of growth. In general, the survival curve that lies above another has a more favorable survival experience (Hosmer et al., 2008) from the bacteria's perspective, not necessarily the patient. The hazard was also calculated as the risk of an event (isolate experiencing growth inhibition during interval (q)) divided by the length of the interval (Hosmer et al., 2008), assuming survival to that point in time. The hazard describes the underlying distribution of survival time and it characterizes how the distribution changes as a function of the covariates (Hosmer et al., 2008).

3.2.3 Discrete Time Survival Analysis

In order for AMR measurements to be used as survival analysis data, the following conditions should apply: 1) There should be a target event whose occurrence was under study; in our study, a target would be the occurrence of an MIC dilution recorded for each isolate (otherwise, right-censored). 2) There should be a beginning time where all isolates are susceptible to a target antimicrobial at very low concentration; in our study, theoretically each isolate that was naïve to the target antimicrobial would

fail to grow (or grow) in the lowest dilution but grow at zero concentration. 3) There should be a metric for clocking time where the event occurrence was measured; in our study, this was the number of dilutions (intervals) between the very lowest dilution and the MIC dilution (or, highest concentration in the assay for right-censored observations). The order in which events (in this analysis, MICs) occur is critical in survival analysis. Furthermore, handling of ties, i.e. two or more events occurring at the same time, may present a problem when comparing among groups with hypothesized differences in hazard. Although ties can be handled using different approaches in Cox regression, using survival analysis to assess differences in MIC distribution is especially problematic because of the very few possible events (MIC values). Hence, discrete-time survival analysis provides an alternative to handle data with a great many ties.

Survival analysis models are also named failure-time models and they calculate average time to occurrence of an event (MIC dilution). The basic model is:

$$\text{Ln}\,[E(T|X_1 = x_1] = \alpha - \beta_1 x_1 \qquad [1]$$

Here, α is the average log incidence time in a subpopulation where $X_1=0$, and $-\beta_1$ is the difference in average log incidence times when comparing the subpopulation with $X_1=x_1+1$ to the population with $X_1=x_1$. Here, the sign for β_1 is reversed, whereas in a normal regression, positive β_1 corresponds to harmful effects from increasing X_1, and negative values are beneficial effects (i.e. if T is death and there is a positive β_1, an increase in X_1 will be associated with an earlier death) (Hosmer et al., 2008). A more

common interpretation of these models (i.e. Cox model or proportional hazards model) is a model for the risk of the event up to each point in time (or the rate at each point in time). "In statistical theory, the assumption is made that, at each time t, the rate $I(t;x_1)$ approaches a limit $h(t;x_1)$ as delta goes to zero" (Hosmer et al., 2008). This limit is usually called the hazard or intensity of the outcome at time (t).

$$h(t;x_1) = \exp(\beta_1 x_1)\, \lambda_0(t) \qquad [2]$$

The hazard is a conditional probability in that an event can occur in any time interval, only so long as it has not occurred in an earlier time interval. There are three assumptions inherent to the population represented by the discrete-time hazard model. First, there is a postulated logit hazard function for each value of the predictor. For a dichotomous variable such as herd type (organic vs. conventional in our study), there are two hazard functions. The second assumption is that each hazard function has an identical shape. The third assumption is that the distance between each logit hazard function is identical in each time period (Singer and Willett, 2003).

3.2.4 Data structure for DTSA

In order to use a discrete time survival analysis, the data must be converted from subject-period format (isolate number and MIC value) into subject-period time data. The subject-period data set was expanded so that each isolate was represented in multiple lines. Each line represents the dilutions up to and including the isolate's reported MIC value. Each time period or MIC interval was represented as C1 through C15. For

example, an isolate with an MIC value at the third concentration (or third interval after log base 2 transformation) would have 3 lines of data, and the covariates would be the same for each line. The interval indicator, j, would take on values 1, 2, 3 (i.e., each MIC value). The binary outcome (y) would be zero for the first 2 lines then one for the third line. This concept is illustrated in **Tables 3.1 and 3.2** where Table 3.1 is the original data format and Table 3.2 represents the modified data format as described above. The estimated coefficients for covariates would be presented and interpreted in the same manner as a fitted proportional hazards model (Hosmer et al, 2008). The time indicators are included as follows where (D) is the 'Jth' dummy variable for the time indicator or number of MIC categories:

$$\text{Logit } h(t_j) = [\alpha 1 D_1 + \alpha_2 D_2 + \ldots + \alpha_J D_J] + [\beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_P X_P] \qquad [3]$$

The left side of the function is the link function. The right side includes the *alphas*, which are multiplied by their time or category indicators (D). These are multiple intercepts by period (MIC value) and are the baseline logit hazard function. The β's represent the effect of one unit difference in the event while controlling for other predictors (Singer and Willett, 2003). The set of the multiple intercepts (α's) estimate the baseline logit hazard function and are not interpretable.

Table 3.1. The original "isolate level" MIC data set for *Campylobacter* isolates' MIC values from antimicrobial-free and conventional swine farms. Six isolates were selected.

				MICs (ug/ml)														
Isolate ID	MIC Cat.	Censor	Farm type	<= 0.02	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	>= 256
1	4	0	0	1	1	1	1	.	.	.	.	.	.	.	.	.	.	.
2	15	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
3	5	0	0	1	1	1	1	1	.	.	.	.	.	.	.	.	.	.
462	5	0	1	1	1	1	1	1	.	.	.	.	.	.	.	.	.	.
463	.	0	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
464	8	0	1	1	1	1	1	1	1	1	1	.	.	.	.	.	.	.

Table 3.2. Converted "MIC-period" data set for *Campylobacter* isolates on antimicrobial-free and conventional swine farms.

Isolate Id	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	Farm type	Y
1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1

Table 3.2 (continued)

Isolate Id	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	Farm type	Y
3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
462	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
462	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
462	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
462	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
462	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1
463	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
464	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
464	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
464	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
464	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
464	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
464	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
464	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
464	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1

C = interval 1 through 15 (i.e. C1 is the lowest MIC dilution); Y is outcome;

A discrete time hazard model based on a logit transformation assumes proportional odds. Therefore, a complementary log-log transformation was used to create a proportional hazards model (Singer and Willett, 2003) and to account for the fact that the fitted hazard values are bounded from [zero to 1].

Clog-log=log(-log (1-probablity)) [4]

The clog-log transformation maps probability onto a new scale with no upper or lower limit which is similar to the logit link. Using a clog-log link makes the DTSA more similar to the Cox regression which analyzes data in continuous time scale and which also has a proportionality assumption in the hazards and not the odds (Singer and Willett, 2003).

The proportional odds assumption means that each covariate has an identical effect in every time period under study. In the case of our study, we asked, “does the effect of herd type (antimicrobial free vs. conventional farms) on the value of the MICs from low to high dilutions differ?” The proportional odds assumption was assessed graphically to compare the hazards (logit) graph of the two levels of the covariate. In a DTSA model, this assumption could be relaxed by including the interaction term of time (here MIC) and the covariate. In our study, the interaction term was MIC*herd type and could be tested by comparing the deviance between the main effects model and the one with the interaction term. Nested models were compared and the one with the lower deviance was preferred model based on fit of the model.

3.3. Results

3.3.1 Descriptive results

The distribution of the log base 2 transformed MIC values for each antimicrobial-bacteria combination are presented for both conventional farms and antimicrobial-free farms in **Figures 3.1-3.6**. Gentamicin-*E. coli*, chloramphenicol-*E. coli*, and gentamicin-*Campylobacter* resembled normal curves, while azithromycin-*Campylobacter* and ampicillin-*E. coli* were bimodal with two local maxima; one at a relatively low MICs and a second at the highest possible MIC ($\geq$ 256µg/mL). The tetracycline-*E. coli* MIC distribution was uniform in appearance. The large proportion of isolates in the highest dilution indicated that each of these had a large percentage of isolates whose growth was not inhibited by exposure to the respective antimicrobial drugs. In general, the shapes of the MIC distribution between antimicrobial-free and conventional farms for each antimicrobial-bacteria pair looked similar.

3.3.2 Life tables and survival curves

Life tables were constructed for each antimicrobial bacteria combination (Appendix D). Five of the six antimicrobial-bacteria combinations had isolates in the highest dilution within both production types. Therefore, those MIC distributions are considered right censored. The gentamicin-*Campylobacter* combination did not have isolates in the highest dilution using Etest®.

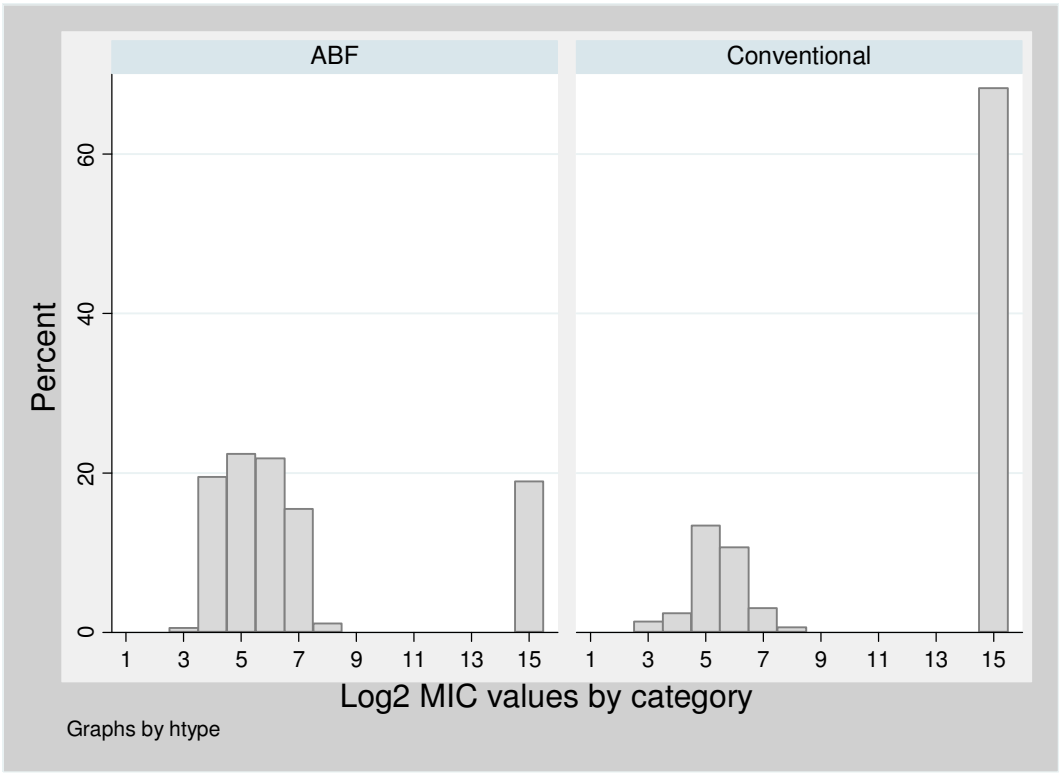


Figure 3.1. Probability (expressed as percentage) distribution of the log2(MIC) values of azithromycin among 464 *Campylobacter* isolates from swine on antimicrobial-free (*n*=174) and conventional (*n*=290) farms. The CLSI interpreted breakpoint was ≥2 ug/mL (log2-MIC category≥8).

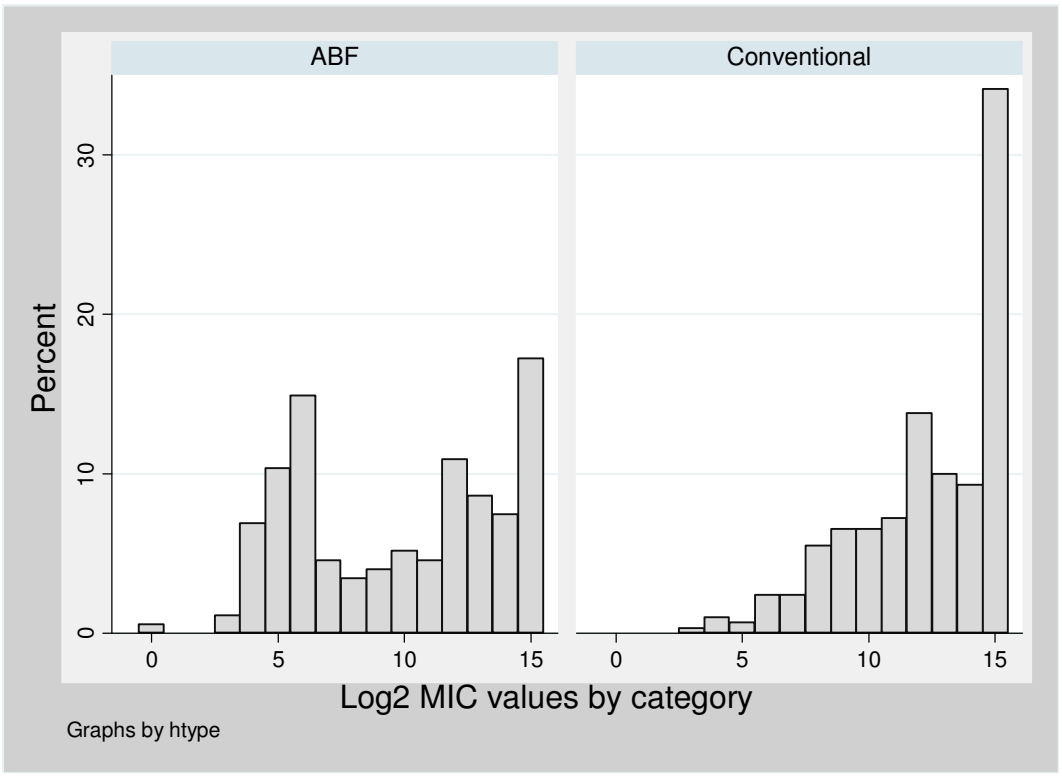


Figure 3.2. Probability (expressed as percentage) distribution of the log2(MIC) values for tetracycline in 464 *Campylobacter* isolates from swine on antimicrobial-free(*n*=174) and conventional (*n*=290) farms. The CLSI interpreted breakpoint was (≥16 ug/mL) (log2-MIC category≥11).

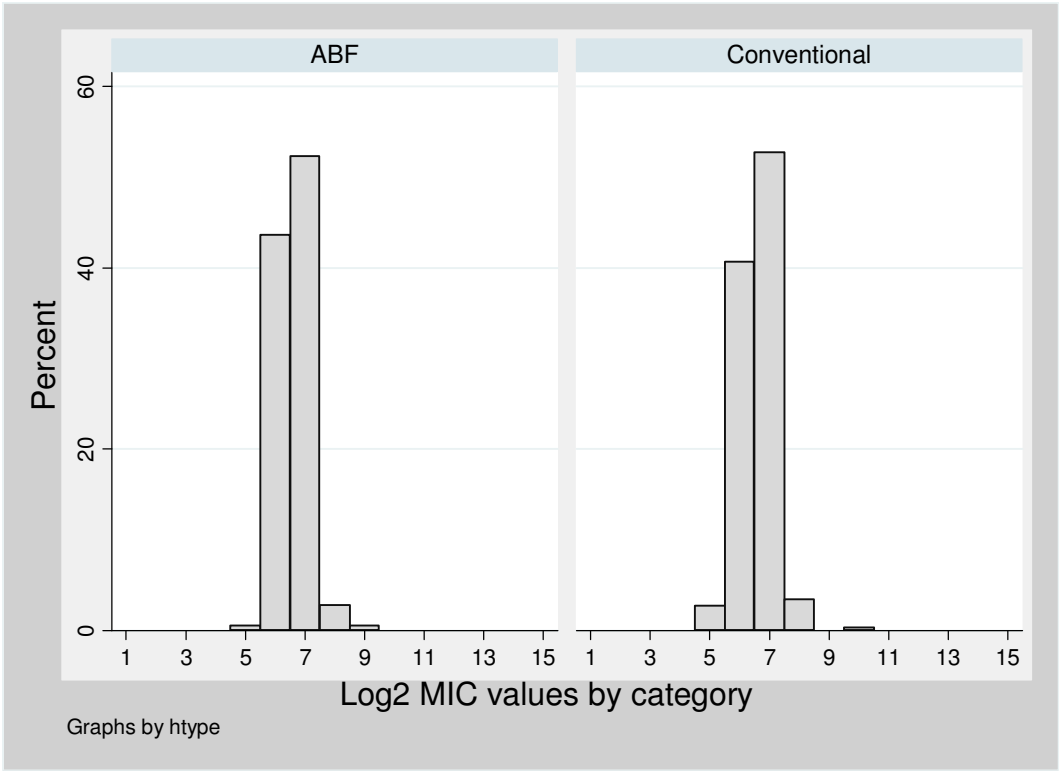


Figure 3.3. Probability (expressed as percentage) distribution of the log2(MIC) values of gentamicin in 464 *Campylobacter* isolates from swine on antimicrobial-free (*n*=174) and conventional (*n*=290) farms. The CLSI interpreted breakpoint was (>=16 ug/mL) (log2-MIC category≥10).

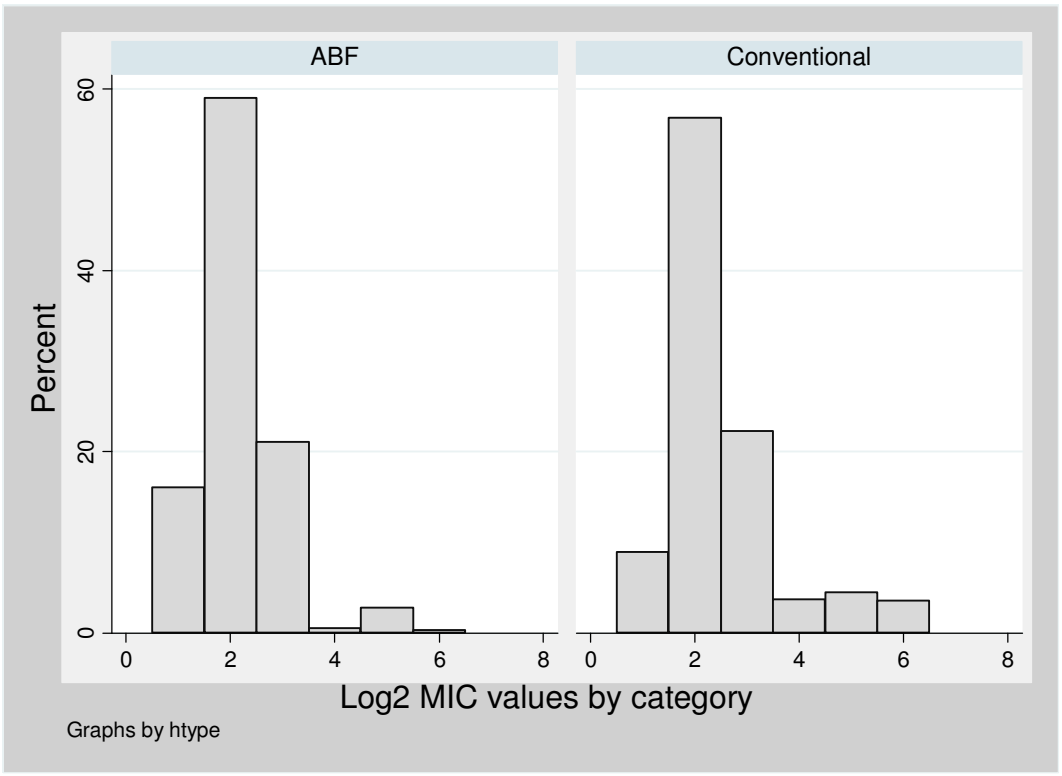


Figure 3.4. Probability (expressed as percentage) distribution of the log2 (MIC) values of chloramphenicol in 1,381 *E. coli* isolates from swine on antimicrobial-free (*n*=498) and conventional (*n*=883) farms. The CLSI interpreted breakpoint was (≥32 ug/mL) (log2-MIC category≥5).

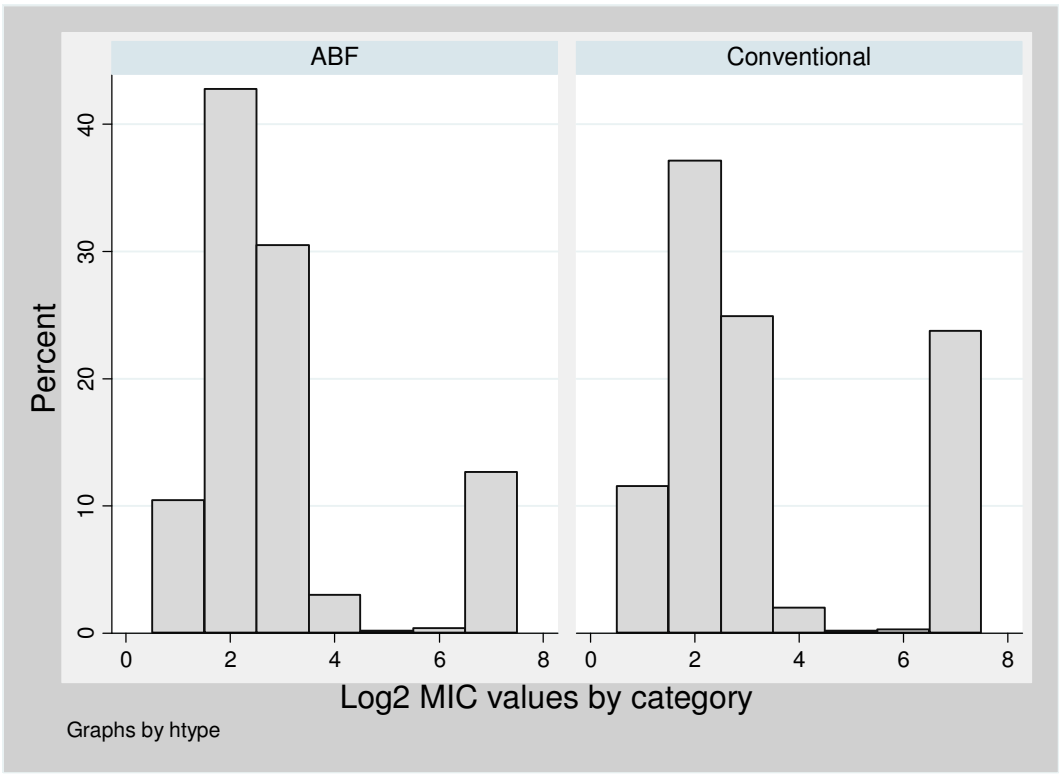


Figure 3.5. Probability (expressed as percentage) distribution of the log2 (MIC) values of ampicillin in 1,381 *E. coli* isolates from swine on antimicrobial-free (*n*=498) and conventional (*n*=883) farms. The CLSI interpreted breakpoint was (≥32 ug/mL) (log2-MIC category≥6).

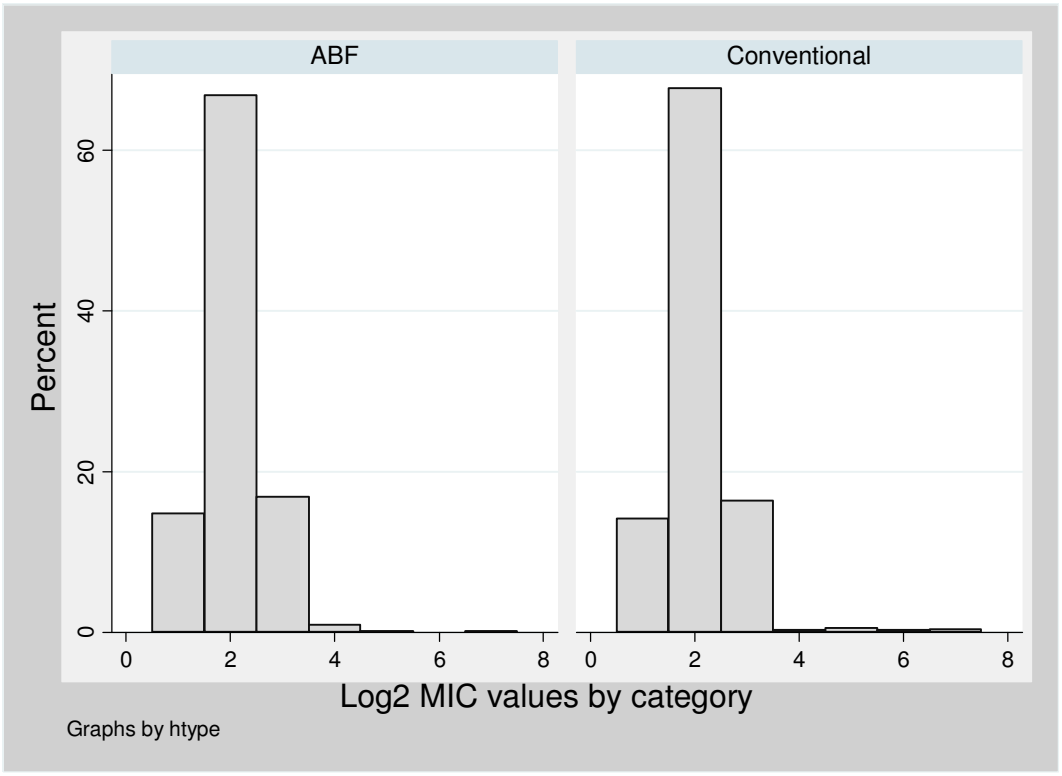


Figure 3.6. Probability (expressed as percentage) distribution of the log2 (MIC) values of gentamicin in *E. coli* isolates from swine on antimicrobial-free (*n*=498) and conventional (*n*=883) farms. The CLSI interpreted breakpoint was (≥32 ug/mL) (log2-MIC category≥6).

Kaplan-Meier survival estimates were constructed graphically by herd type (**Figures 3.7-3.12**). The survival curve for azithromycin-*Campylobacter* (**Figure 3.7**) showed that a larger proportion of the isolates grew at the highest azithromycin concentration in conventional farms compared to antimicrobial-free farms. The survival curves for tetracycline susceptibility of *Campylobacter* were of stair-step shape across all possible dilutions and which were parallel between production types. However, the gentamicin survival curve for both *Campylobacter* and *E. coli* had either no isolates or a few isolates, respectively, that survived the highest concentration (were right-censored). The shape of the survival curve between production types was similar for the gentamicin-*Campylobacter* and gentamicin-*E. coli* combinations. The survival curve of *E. coli* isolates that were exposed to ampicillin was similar between production types, but there was a higher proportion of isolates at the highest dilution in conventional farms.

3.3.3 Discrete time survival analysis

The original dataset has one line for each isolate and its respective MIC value (**Table 3.1**). Six isolates were included in this example including three each from antimicrobial-free and conventional farms. One isolate in this example was censored because the MIC category was ≥256 ug/mL. For use in the DTSA model, these six isolates were converted into a subject-period data set (**Table 3.2**).

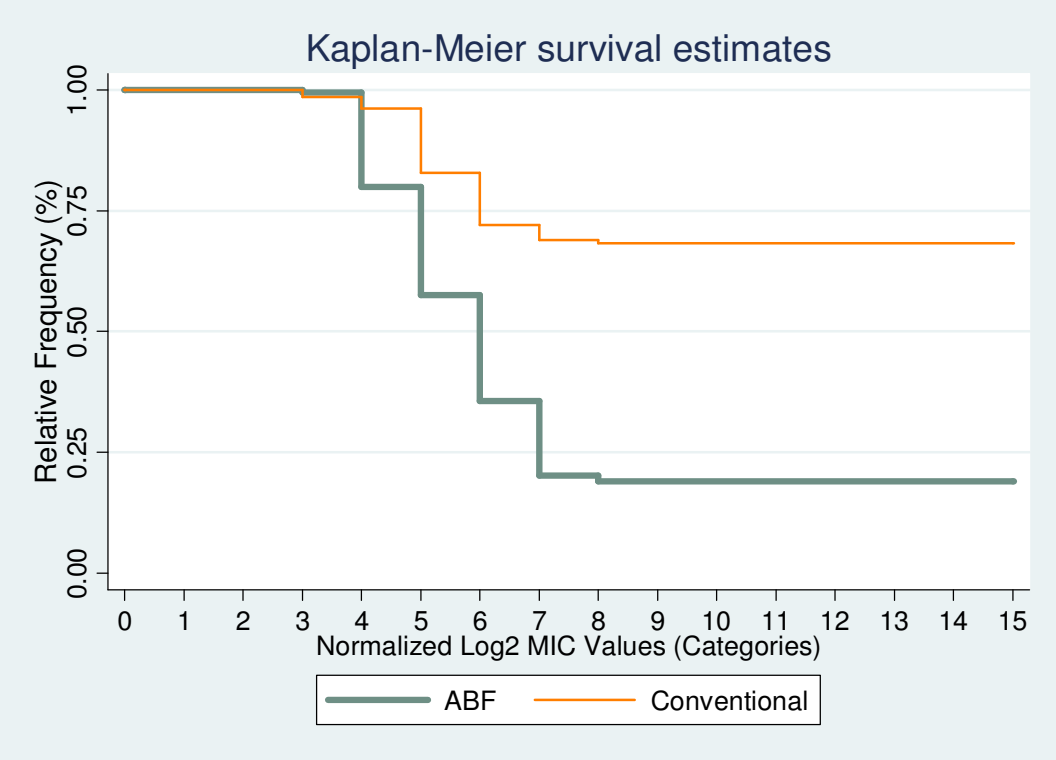


Figure 3.7. Estimated Kaplan-Meier survival curves for log2-transformed MIC values for azithromycin in 464 *Campylobacter* spp. isolates from antimicrobial-free (ABF) and conventional swine herds.

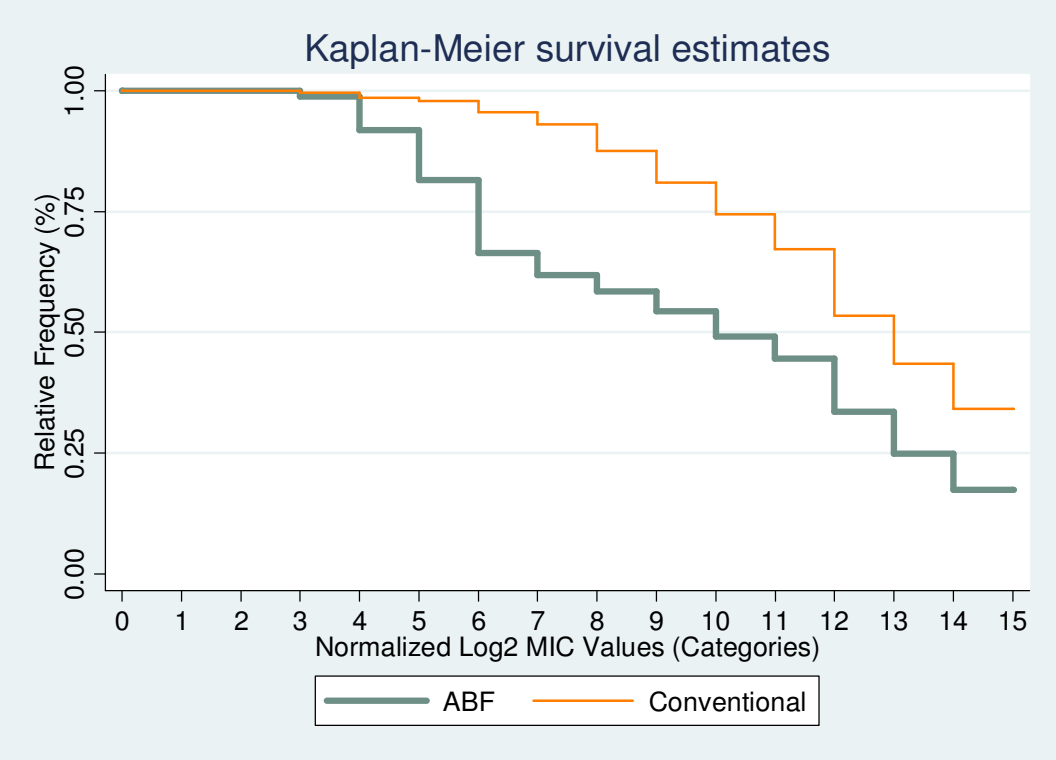


Figure 3.8. Estimated Kaplan-Meier survival curves for log2-transformed MIC values for tetracycline in 464 *Campylobacter* spp. isolates from antimicrobial-free (ABF) and conventional swine herds.

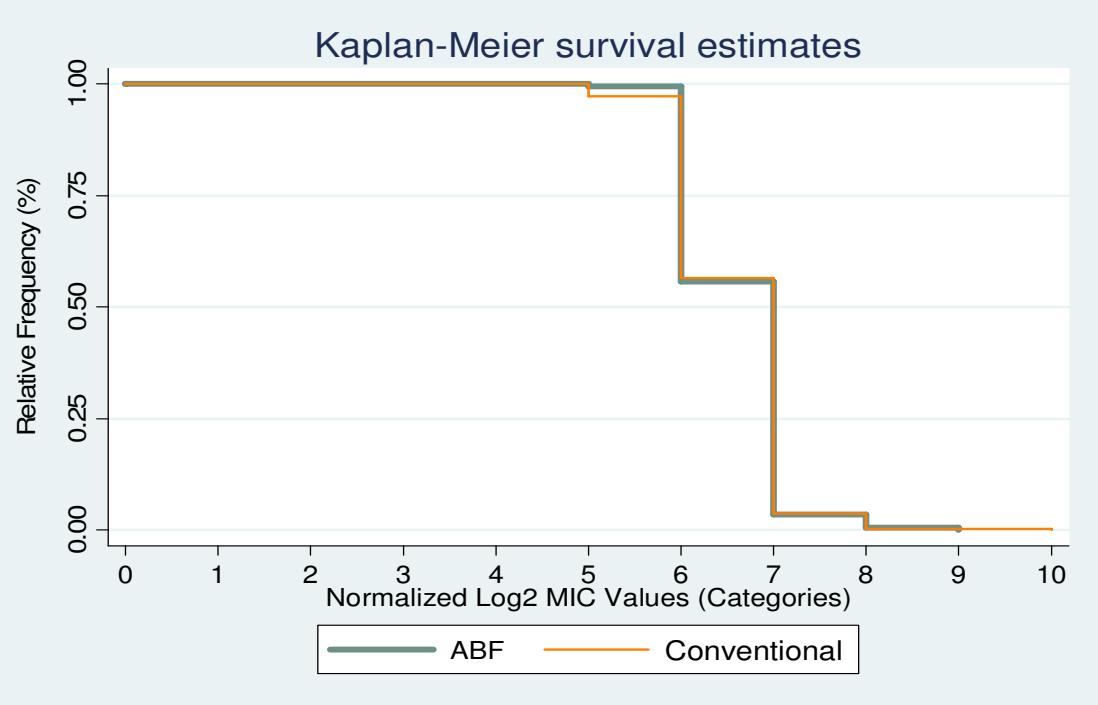


Figure 3.9. Estimated Kaplan-Meier survival curves for log2-transformed MIC values for gentamicin in 464 *Campylobacter* spp. isolates from antimicrobial-free (ABF) and conventional swine herds.

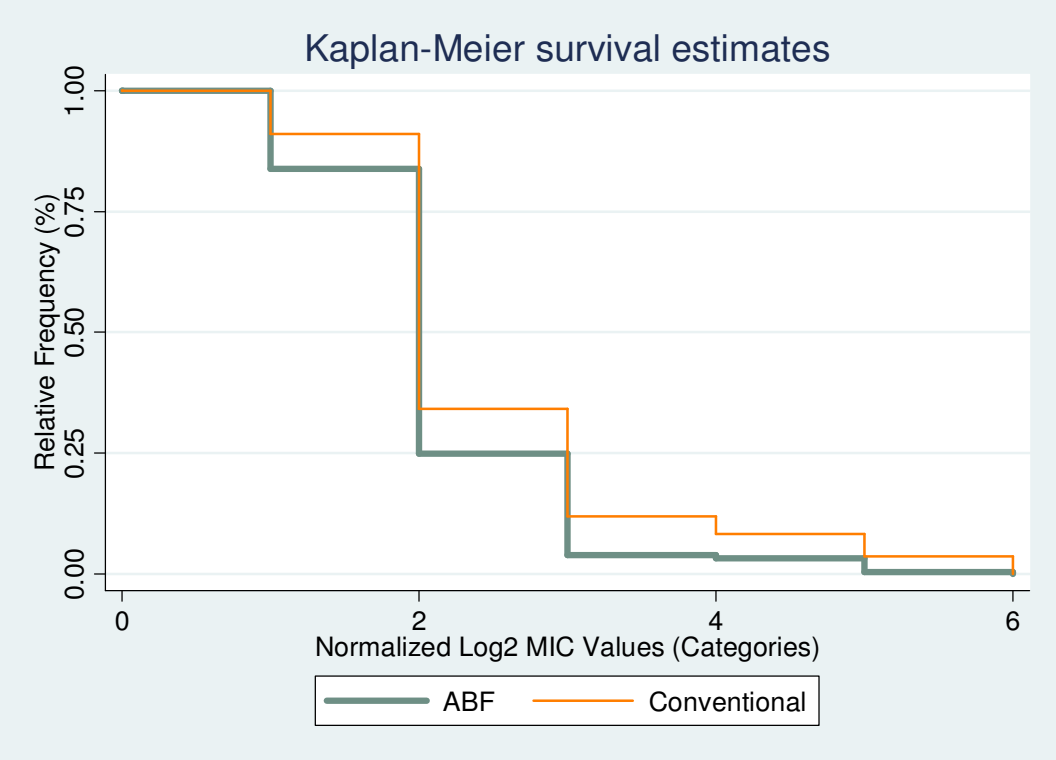


Figure 3.10. Estimated Kaplan -Meier survival curves for log2-transformed MIC values for chloramphenicol in 1,381 *E. coli* isolates from antimicrobial-free (ABF) and conventional swine herds.

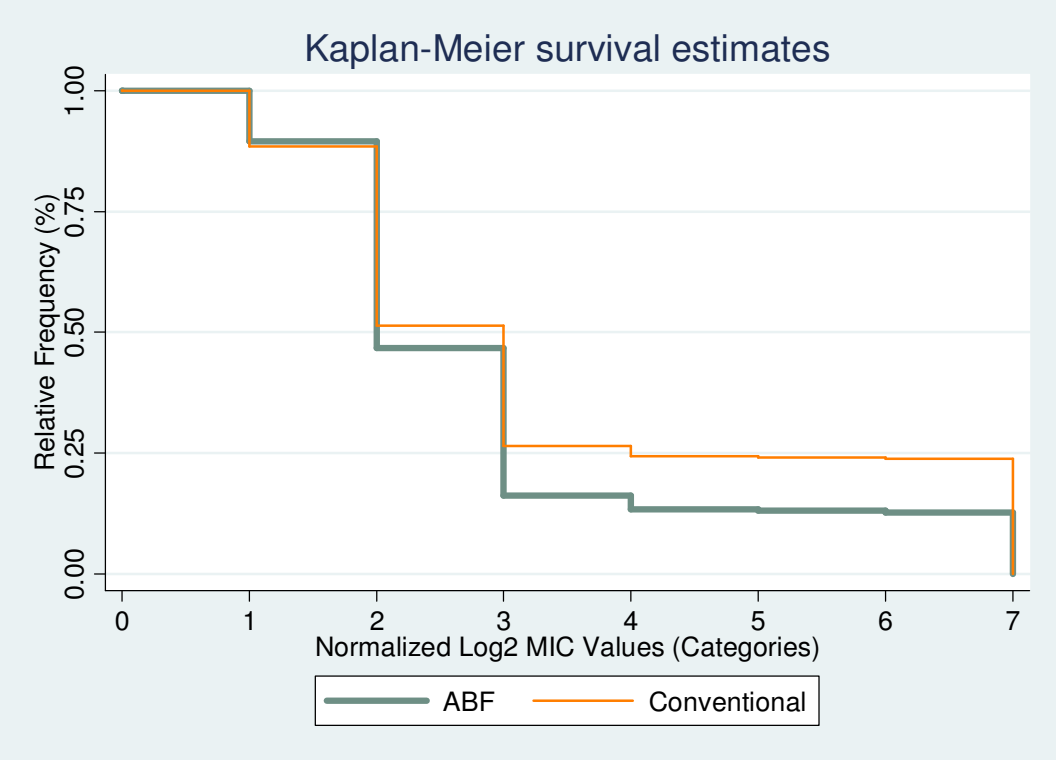


Figure 3.11. Estimated Kaplan-Meier survival curves for log2-transformed MIC values for ampicillin in 1,381 *E. coli* isolates from antimicrobial-free (ABF) and conventional swine herds.

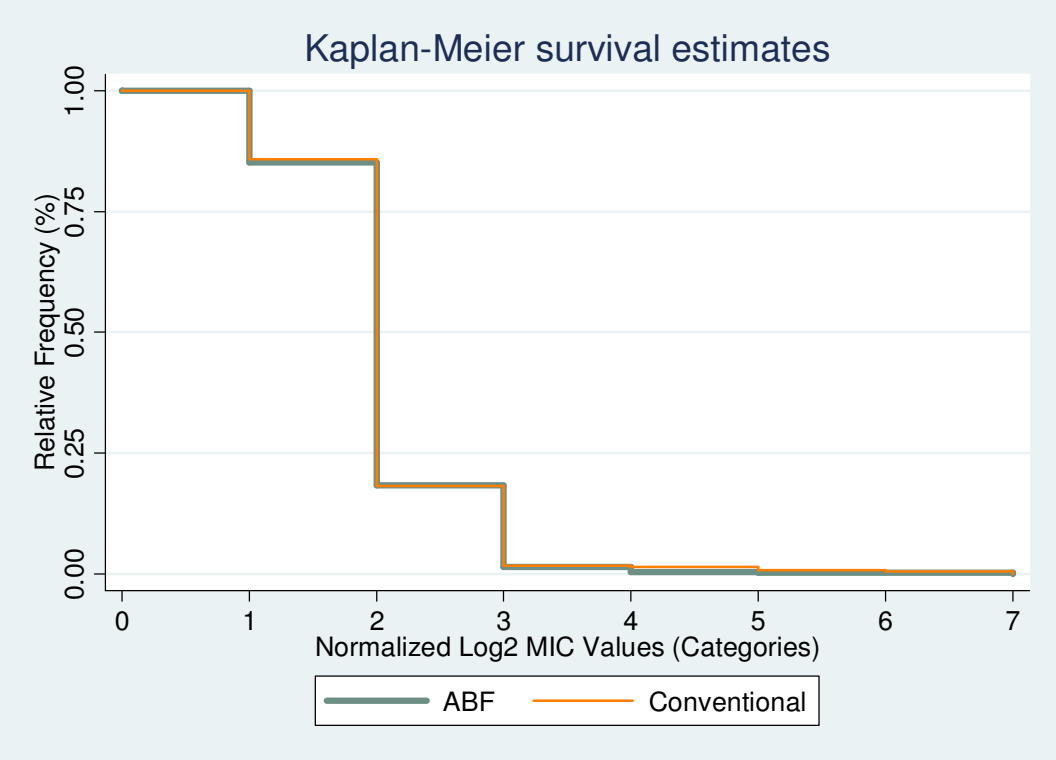


Figure 3.12. Estimated Kaplan-Meier survival curves for log2-transformed MIC values for gentamicin in 1,381 *E. coli* isolates from antimicrobial-free (ABF) and conventional swine herds.

A DTSA model using clog-log link function was used to analyze MIC distributions among six antimicrobial-bacteria combinations. An interaction term between MIC dilution and herd type was included to relax the proportionality assumption inherent to survival analysis data (**Table 3.3**). The deviances between the model with the interaction term and the main effects model were compared. The model with the interaction term had a lower deviance so therefore was considered the more parsimonious model. All six models showed a significant difference in the MIC distributions ($P<0.001$) between production types.

3.4. Discussion

In this study we introduced DTSA as a potential modeling framework to be used with data sets where MIC distributions are the outcome. Previously, a population average (GEE) logistic regression model was used to model the proportion of resistant isolates for *Campylobacter* between the two herd types using the same dataset (Rollo et al., 2010). Significant differences in the proportions of resistant bacteria between antimicrobial-free and conventional farms were reported for *Campylobacter* and tetracycline and *Campylobacter* and azithromycin. There was not a difference in proportion of resistant bacteria for *Campylobacter* and gentamicin between production type using GEE (Rollo et al., 2010). In the present study, there was a significant difference ($p<0.001$) in the MIC distributions of all 3 antimicrobial-bacterial combinations. In the DTSA model, the isolates in the highest dilutions were censored.

Table 3.3. Odds ratios and coefficients of the DTSA of the susceptibility of *Campylobacter* isolates and *E. coli* isolates to 6 antimicrobials on antimicrobial-free and conventional swine farms (herd is farm type and referent is ABF farms). The main effects model is listed first and the model which uses an interaction term to account for the proportional hazards assumption is listed second.

Azithromycin-*Campylobacter*	**B (SE)**	**OR**	**95% CI**	**p-value†**
herd (model 1)	-3.19 (0.11)	0.04	0.03-0.05	<0.001
herd (model 2)	2.67 (0.29)	14.4	8.08-25.65	<0.001
interaction	-0.61 (0.05)	0.54	0.49-0.60	<0.001
Gentamicin-*Campylobacter*	**B (SE)**	**OR**	**95% CI**	**p-value†**
herd (model 1)	-2.02 (0.07)	0.13	0.12-0.15	<0.001
herd (model 2)	2.36 (0.63)	10.6	3.07-36.26	<0.001
interaction	-0.66 (0.10)	0.00	0.43-0.62	<0.001
Tetracycline-*Campylobacter*	**B (SE)**	**OR**	**95% CI**	**p-value†**
herd (model 1)	-1.60 (0.08)	0.20	0.17-0.24	<0.001
herd (model 2)	2.14 (0.24)	8.50	5.31-13.58	<0.001
interaction	-0.31 (0.02)	0.73	0.71-0.77	<0.001
Gentamicin-*E. coli*	**B (SE)**	**OR**	**95% CI**	**p-value†**
herd (model 1)	-0.93 (0.04)	0.40	0.36-0.43	<0.001
herd (model 2)	2.25 (0.17)	9.53	6.83-13.31	<0.001
interaction	-1.46 (0.08)	0.23	0.20-0.27	<0.001
Ampicillin-*E. coli*	**B (SE)**	**OR**	**95% CI**	**p-value†**
herd (model 1)	-1.56 (0.05)	0.21	0.19-0.23	<0.001
herd (model 2)	0.06 (0.07)	1.07	0.93-1.23	0.36
interaction	-0.47 (0.02)	0.62	0.59-0.65	<0.001

Table 3.3 (continued)

Chloramphenicol-*E. coli*	**B (SE)**	**OR**	**95%CI**	**p-value†**
herd (model 1)	-1.13 (0.04)	0.32	0.30-0.35	<0.001
herd (model 2)	1.48 (0.12)	4.40	3.48-5.56	<0.001
interaction	-1.01 (0.05)	0.37	0.33-0.40	<0.001

B = Regression coefficient. SE = Standard error. OR = Odds ratio. CI = Confidence interval. †A value of $P \leq 0.05$ was considered significant.

Therefore, these isolates were included in the denominator only, that is, the censored isolates were considered in the probability calculation at each interval but did not become a numerator in the given timeframe or dilution.

The survival curves for *Campylobacter* and *E. coli* and gentamicin appeared to be very similar in shape; however, the distributions were significantly different using DTSA. A possible explanation for this may have been that the number of records in the dataset was inflated in the expansion of data into time-period data. Further studies are needed to investigate this phenomenon.

The proportion of resistant isolates for *E. coli* was also previously modeled using a population average logistic regression model (Bunner et al., 2007). Significant differences between production types were reported for *E. coli* isolates resistant to chloramphenicol and ampicillin, but not gentamicin. In the present study, there was also a significant difference ($P<0.001$) in the MIC distributions for all three antimicrobial-bacteria combinations, including gentamicin. In theory, a model that compares distributions should be able to detect subtle differences in the distributions that do not necessarily depend on the resistance breakpoint. Six antimicrobials were examined that had unique shapes of their distributions to determine if the shape and the proportion of censored data would affect the outcomes. However, it is not possible to compare odds ratios produced by these models to odds ratios in the logistic regression models because of the number of censored isolates. Further analysis of this type of model using simulation studies may provide further guidance on the application of the DTSA model to MIC data.

Discrete time survival analysis models (DTSA) have been used in econometrics and the social sciences. This model was introduced here as an option to analyze MIC data since MIC data are discrete, have many ties, and are often right and left censoring. In the present study, we did not account for left censoring; however, only a few the isolates were left censored for the six antimicrobial drug-bacterial combinations examined.

In summary, right censoring will occur with most diagnostic procedures based on dilutions such as microbroth dilution and Etest®. Most MIC distributions were right censored and all isolates that were not inhibited were grouped in the highest category (i.e. 256 ug/mL). In reality, the true concentration where those isolates would be inhibited is unknown. Left censoring also occurs with these tests because there is a cutoff of measurement at the lowest dilution as well so the outcome may occur at a smaller dilution than what is represented by the test.

In addition to right and left censoring, the data used in the present study were also interval censored. The data used in this study, although values were measured on a continuum, were grouped into discrete intervals (all outcomes that occur in the interval of [64ug/mL to <128ug/mL] were categorized as 64 ug/mL). This would apply to Etest® and most microbiological susceptibility tests since true values are grouped into categories or intervals based on the test methodology. In addition, interval censored data were right censored within each interval. However, addressing censoring within an interval is beyond the scope of this paper.

Besides the censoring considerations, the discrete time interval format of the data created tied outcomes. Hence, the MIC distribution could not be considered to be continuous, which is one of the major assumptions of the Cox proportional hazards model. Cox first proposed a deviation of his proportion hazards model in that not all outcomes would be continuous (Cox, 1972). However, Willet and Singer (2003) have extended this model to be utilized with interval data (Singer and Willett, 2003). Several studies have utilized a Cox proportional hazards model to analyze MIC outcomes (Ray et al., 2006; Stegeman et al., 2006). Stegeman (2006) compared a Cox model to logistic regression. Although several methods to handle ties have been introduced for Cox proportional hazard model, the DTSA model may be superior to Cox proportional hazard model because it doesn't require any assumptions regarding the ties. Tied outcomes should be considered when making a choice between using a Cox proportional hazards model or a DTSA (Singer and Willett, 2003).

When comparing models using a deviance statistic (-2 log likelihood), a small value with a non-significant P-value indicates a good fit of the model. However, if there is a large sample size, the deviance statistic is often significant and thus the null hypothesis of model fit associated with the deviance statistic will be rejected. Akaike's Information Criterion and Bayesian Information Criterion make a correction of the deviance statistic for the number of parameters or for sample size (Singer and Willett, 2003). These criteria can be compared between models that are not nested if used on the same data set. Smaller values indicate better model fit.

A dataset used for DTSA must be expanded or converted into subject-period time data to include one record for each MIC interval and this might be concerning because we inflated the records. That is, there are multiple lines of data per isolate. Multiple lines of data are necessary if we realize that a hazard function describes the conditional probability of event occurrence at time (t) given it has not occurred up to time (t) (Dohoo et al., 2003). Each person or isolate contributes when it is at risk and therefore each isolate is also conditionally independent. Another way to express this data transformation is that DTSA allows the longitudinal progression of the probability that an event will occur.

The final major assumption that needs to be considered in developing the DTSA model is the dependence between outcomes due to clustering or non-independence among isolates within farms. One way to overcome this drawback is to add a random intercept to the model that represents each farm (Rabe-Hesketh and Skrondal, 2005).

This random intercept is referred to as a shared frailty since it accounts for animals from the same farm. Frailty models are complex but they can incorporate an unmeasured 'random' effect into the hazard function to account for heterogeneity among isolates (Hosmer et al., 2008). One problem is that software that fits the proportional hazard model may not have an option for including frailty (Hosmer et al., 2008). Hence, we did not consider dependence between pigs at this time. For more discussion on this subject, refer to Hosmer et al. (2008).

There are other options that have been suggested for analysis of discrete time-series and censored data (Hammel et al., 2006). Hammel suggested removing censored data or replacing censored data with actual values at the tail of the distributions. Using Hammel's suggestion, the model incorporated censored MIC observations into the likelihood function by using the tail probabilities of the error distribution (this preserves the uncertainty of the censored MICs).

Often, epidemiologists will dichotomize MIC data based on breakpoints determined from human drug studies. A limitation of dichotomizing MIC outcomes is that variability in MIC distribution that does not include the breakpoint will not be detected. Furthermore, difference of the distribution that would occur slowly on the scale of genotypic changes are not detected when considering dichotomized outcomes unless a table is included that provides the MIC values for each antimicrobial for antimicrobial-free and conventional farms. When examining longitudinal data, shifts in MICs would be reflected readily since the shifts often include a change encompassing the breakpoint (Stegeman et al., 2006). Stegeman (2006) was particularly concerned with changes in the MIC below the breakpoint because the assumption is that changes occur in a stepwise fashion towards the upper limit. By using a proportional hazard's model, subtle changes are detected, whereas by dichotomizing the data, that information would be lost. The analysis of MIC data by logistic proportional hazards model provided a more sensitive test for detecting incremental differences.

3.5. Conclusion

We have described the DTSA model and how it can be used to model MIC data. The characteristics of MIC data including right censoring and discrete intervals can be accounted for with the assumptions of a DTSA model. The right censored isolates are included only in the denominator since their true interval is not definable within the limits of the Etest®. The DTSA model should in theory be a better option for modeling MIC data as compared to Cox regression. However, some issues in respect to e.g. the

impact (if any) of extending the data for an observation to multiples lines on test statistics, accounting for hierarchical data, and an additional measure of ‘fit’ must be investigated further.

4. HERD-LEVEL RISK FACTORS ASSOCIATED WITH ANTIMICROBIAL RESISTANCE IN *E. coli* AND *Campylobacter* SPP. ON ANTIMICROBIAL-FREE AND CONVENTIONAL SWINE FARMS IN THE U.S.

4.1. Introduction

Antimicrobial resistance (AMR) in agricultural systems is an ongoing concern to both human and animal health (Molbak, 2004; Mathew et al., 2007). Both commensal and pathogenic bacteria obtained from swine farms, including *Campylobacter* spp. *Salmonella* spp., and *Escherichia coli* (Rollo et al., 2010, Taylor et al., 2009; Bunner et al., 2007; Dunlop et al., 1998) may be resistant to a large range of antimicrobial drugs. Antimicrobial-resistant bacteria of food animal origin are of concern because they may be transmitted through the food chain to humans. Such resistant bacteria of food animal origin may cause human infections that are difficult to treat, and they may exchange genetic resistance determinants with commensal or pathogenic bacteria already in the human gut.

E. coli are present in the gastrointestinal tract of most warm-blooded animals as commensals (Hartl and Dykhuizen, 1984). *E. coli* are also present in the environment and can serve as reservoirs for resistance genes that can be transferred to pathogenic bacteria (Sunde et al., 1998; Windfield and Groisman, 2003; Anderson and Sobsey, 2006). However, the actual transfer of resistant genes from commensal bacteria to pathogenic bacteria has not been thoroughly investigated *in vivo* (Mathew et al., 2007).

The theory suggests that the exposure of commensal bacteria to antimicrobial drugs can lead to an increase in prevalence of genes carried on mobile genetic elements such as plasmids, integrons, and transposons (Lees et al., 2008).

In most cases, resistant bacterial strains are associated with the type of antimicrobial drugs used both historically and in the present on the farm (Harada et al., 2008; Rosengren et al., 2009; Varga et al., 2009); however, there are often clones that are resistant to antimicrobial drugs for which there is no history of use on the farm (Thakur and Gebreyes, 2005). For example, bacteria that are resistant to fluoroquinolones are apparent on some poultry farms that have never used drugs in this group (Taylor et al., 2009). In countries where some antimicrobial drugs are now banned, antimicrobial resistance is still present to these antimicrobial drugs (Bischoff et al., 2002; Harada et al., 2006). Furthermore, on antimicrobial-free farms resistance is present, although at lower proportions as compared to conventional farms (Sato et al., 2004a; Halbert et al., 2006; Luangtongkum et al., 2006; Bunner et al., 2007; Rollo et al., 2010). A number of authors have addressed the multitude of mechanisms promoting AMR persistence and the complex interactions between antimicrobial drugs and bacterial species (Engberg et al., 2001; Andersson, 2003; Alfredson and Korolik, 2007).

Co-resistance and cross-resistance are two mechanisms that may help explain the persistence of resistance to antimicrobial drugs that have never been, or are not currently being, used on a farm. Use of one antimicrobial drug can co-select for resistance to other antimicrobial drugs in the absence of use of these other drugs. This phenomenon is referred to as co-selection. In other words, the use of an antimicrobial drug which causes

the selection of a resistance determinant for a particular drug may result in selection of a resistance determinant for another antimicrobial drug. Co-resistance, also called associated resistance, is due to the co-existence of resistance-determinants in the same bacterial strain causing resistance to different antimicrobial drugs. For example macrolides, lincosamides, and B streptogramins act on bacterial ribosomes, and methylation of a single adenine residue in 50S rRNA confers high-level resistance to the three antimicrobial classes despite differences in their chemical structure (Roberts et al., 1999).

Cross-resistance occurs when one gene confers resistance to more than one type of antimicrobial drug (Guardabassi and Kruse, 2008). Both co-resistance and cross-resistance occur in most bacterial populations including *Campylobacter, Salmonella,* and *E. coli*. Besides co-and cross-resistance of bacteria during antimicrobial drug use, other herd-level management factors may affect the levels and patterns of antimicrobial resistance on swine farms. We have previously shown that the prevalences of resistance to some antimicrobial drugs were lower in *E. coli* and *Campylobacter* isolated from the feces of pigs on antimicrobial-free farms compared to conventionally managed farms (Bunner et al., 2007; Rollo et al., 2010). However, there may be other management practices on these types of farms that are associated with the occurrence of antimicrobial resistance. Examples of such management practices may include biosecurity practices, disease history, preventive medicine practices, other farm management practices, and vaccine administration. The goal of this study was to identify potential herd-level risk

factors associated with AMR among *Campylobacter* and *E. coli* in pigs from antimicrobial-free and conventional swine farms in the Midwest.

4.2. Materials and Methods

4.2.1 Study design and sample collection

This study was a part of a larger study undertaken in 2002-2003 in the Midwestern United States. A cross sectional design was used to collect data from 35 antimicrobial-free and 60 conventional finishing swine farms. The methods for herd selection, sample collection, and bacterial isolation, and susceptibility testing have been previously described in detail (Bunner et al, 2007, Rollo et al, 2010). In summary, antimicrobial-free farms were selected from membership lists of 2 cooperatives that produced pigs without the use of antimicrobial drugs. Farmers were contacted by telephone and asked if they would participate in the study. Conventional farms were selected on the basis of their close geographic proximity to the antimicrobial-free farm and the number of slaughter pigs produced per year. The total number of pigs marketed per year was used as a surrogate for herd size. Participating swine farms were visited once in 2002-2003, and feces were collected from 15 healthy finisher pigs per farm; however, on one farm only 12 finishers were sampled.

4.2.2 Bacterial isolation and antimicrobial susceptibility testing of Campylobacter

Each fecal sample was cultured for isolation of *Campylobacter* spp. and subsequently screened with a panel of antimicrobials to determine resistance prevalence and patterns. Of the 512 *Campylobacter* isolates that were recovered, 174 and 290 isolates were available for susceptibility testing from 30 of 35 antimicrobial-free farms and 55 of 60 conventional farms, respectively.

For specifics on isolation and identification of *Campylobacter*, please refer to Section 2. Antimicrobial susceptibility testing was performed using gradient disk diffusion strips (Etest®) according to the manufacturer's instructions and as described by Sato et al. (2004a) and Rollo et al. (2010) (AB Biodisk, Piscataway, NJ). Susceptibility results were interpreted as described in Sato *et al*. (2004a). Six antimicrobials were tested: azithromycin, erythromycin, ciprofloxacin, nalidixic acid, gentamicin, and tetracycline. Data on azithromycin and tetracycline were included in this risk-factor study as single models and all six antibiotics were considered in the multidrug resistant model (see Section 2 for other dilution ranges and breakpoints of the additional antimicrobials). The dilution ranges were 0.016-256 µg/mL for azithromycin and 0.016-256 µg/mL for tetracycline. Etest® values were expressed on a quasi-continuous scale with intermediate values present between each set of $\log_2$ dilutions; however, intermediate values between $\log_2$ dilutions were rounded up to the higher $\log_2$ dilution during post-study data management, as recommended by the manufacturer. The resistance breakpoints used by the National Antimicrobial Resistance Monitoring System in 2003 were adopted (CDC, 2003), since those were the applicable ones for this

time period and CLSI had not yet defined breakpoints for *Campylobacter.* The resistance breakpoints were ≥ 2 μg/mL azithromycin and ≥ 16 μg/mL for tetracycline. Results of susceptibility testing are reported both as MIC distributions and proportions of resistant and susceptible isolates according to CLSI performance standards (CLSI, 2008).

4.2.3 Bacterial isolation and antimicrobial susceptibility testing of E. coli

For the *E. coli* isolates, standard isolation and identification techniques were performed as described by Bunner et al, 2007. Of the 1,422 fecal samples collected, 1,381 *E. coli* isolates were recovered so that 498 and 883 isolates were available for susceptibility testing on all 35 antimicrobial free farms and all 60 conventional farms, respectively.

In addition, susceptibility to 14 antimicrobial agents was determined for each *E. coli* isolate using a microbroth dilution test (Sensititre panel CMV7CNCD, Trek Diagnostics, Westlake, OH). However, only data on five antimicrobial agents were used as single models: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. All 14 antimicrobials were used in the multidrug resistance model. The dilution ranges for the four drugs were: ampicillin (1 to 32 μg/mL), chloramphenicol (2 to 32 μg/mL), streptomycin (32 to 64 μg/mL), sulfamethoxazole (16 to 512 μg/mL), and tetracycline (4 to 32 μg/mL). The resistance breakpoints for the four antimicrobial drugs were: ampicillin (≥32 μg/mL), chloramphenicol (≥16 μg/mL), streptomycin (≥64 μg/mL), sulfamethoxazole (≥512 μg/mL), and tetracycline (≥16 μg/mL) (see Bunner et al., 2007 for other antimicrobials and breakpoints used). Isolates with an MIC greater

than or equal to the breakpoint MIC were classified as resistant and the MIC breakpoints were determined using data from human studies (Bunner et al., 2007).

4.2.4 Herd management practices and data collection

At the time of the sampling, a questionnaire modeled after the National Health Animal Health Monitoring System – Swine 2000 study was administered to each farm manager (USDA, 2001). The questionnaire was divided into the following sections: herd information, environment of pigs, medication history, preventive medicine, biosecurity, disease history, and production performance (Appendix E). Types of antimicrobial drugs used were also collected; however, antimicrobial free farms did not use antimicrobial drugs so these data were excluded from further analysis. Data on production performance were also excluded because they were only available for some of the farms (some producers did not record production data routinely). In addition, data on gilt, sow, and nursery pig management practices were excluded. Herd management practices as defined for this project include the housing environments of pigs and describe type of house, ventilation, bedding, floor type, and flooring.

Independent variables (risk factors) were constructed from the questionnaire data at the farm level. Variables that were excluded were those with high numbers of missing values, low variability (less than 20% difference between farm type), or unclear answers (Dohoo et al., 2003). Appendix F lists the variables that were recorded for the study. For example, some biosecurity measures that captured what visitors had to do when visiting a farm were collapsed into fewer variables to account for minimal variability between variables. Specifically, only a few farms required visitors to wait at least 24 hours from

the last contact with pigs to enter a farm; hence, this variable was combined with a variable measuring whether or not visitors had to "shower in" before entering the farm. Data were summarized by calculating descriptive statistics including, medians, standard deviations (SD), and ranges when indicated.

Data on *Campylobacter* and *E. coli* isolates, AMR, and variables created from the questionnaire were compiled in a commercially available database software program (Microsoft Access, 2003, Microsoft Corp, Redwood, WA). The dataset was checked for proper coding and distribution of values and then was imported into another software (STATA, version 10.0, StataCorp, College Station, TX) package for statistical analysis. Data validation was conducted by examining a selection of questionnaires and cross-checking the database to ensure proper coding and to check for potential errors during data entry.

4.2.5 Statistical methods and model building

In the initial analysis, the dependent variable was the proportion of bacterial isolates that were resistant to a specific antimicrobial drug. The dependent variable was measured at the individual animal whereas the independent variables were measured at the farm level. A total of seven bacteria-antimicrobial combinations were analyzed as the outcome in seven separate models: *Campylobacter*-azithromycin and -tetracycline, *E. coli*-ampicillin, -chloramphenicol, -streptomycin, -sulfamethoxazole, and -tetracycline.

All independent variables were screened initially to allow evaluation of simple associations with each of the outcome variables by calculating an odds ratios (OR) and associated 95% confidence interval. Furthermore, herd-type (antimicrobial-free and

conventional) was included in all models, since it was the main exposure variable for this study. During the initial screening process, a population-average logistic regression model involving a generalized model framework with a logit link and binomial error distribution, with generalized estimating equation involving an exchangeable working correlation structure and semi-robust variance estimator, was used to determine the potential association between the proportion of resistance for each of the antimicrobial agents, herd-type (forced into each model), and the additional variable that was being screened (Hosmer and Lemeshow, 2000; Dohoo et al., 2003). Screening of variables was conducted in subsets (preventive medicines, biosecurity, vaccine status, production management, and disease history). A level of significance of 0.25 was used to screen variables. The preliminary screening process was used as an approach to eliminate the problem of multicollinearity (Dohoo et al., 1996).

Multicollinearity among categorical predictor variables was also checked by considering the associations between each pair of the categorical predictor variables within each subset of management variables using the Pearson chi-square test of independence (Agresti, 1996). A significance level of less than 0.05 resulted in the rejection of the null hypothesis of independence. A pair-wise calculation of Spearman rank correlations was used to investigate collinearity between predictor variables within each subset of management variables. When two potential risk factors were highly correlated (correlation coefficient >0.7), only one variable was used in the multivariate analysis.

Those variables that met the criterion of independence were further screened for inclusion in a multivariable model. Within each subset of management variables, the criterion for inclusion in the final multivariate model was a level of significance of 0.l0 or less. Inclusions of variables for multivariable models were selected by using a backwards selection process (Hosmer and Lemeshow, 2000). After deletion of non-significant variables, eliminated variables were added in a forward selection process to check that a variable was not prematurely removed ($p<=0.05$) to obtain the multivariate model. Dichotomous and nominal ordinal variables were assessed using a generalized Wald test (Hosmer and Lemeshow, 2000). Standard errors of the coefficients were examined to determine any unstable coefficients. Once the main effects model was obtained, two-way interactions were tested. An interaction term was added to the model and retained if it was significant ($P \leq 0.05$). Potential confounding variables were assessed by comparison of the differences in the regression coefficients of the main exposure variable (herd-type) with and without the presence of the potential confounder in the model. If there was a change of 20% or more, then the confounding variable was forced in the model.

Multidrug resistance (MDR) was defined as resistance to two or more antimicrobial drugs. Associations between MDR and herd management factors were determined for *Campylobacter* spp. and *E. coli* separately (*E. coli*-MDR and *Campylobacter*-MDR). Bunner et al. (2007) analyzed the proportions of MDR for 14 antimicrobial drugs for *E coli* isolates and Rollo et al. (2010) analyzed MDR proportions for six antimicrobial drugs for each *Campylobacter* isolate.

Multi-bacterial-antimicrobial resistance (MBAR) was considered for fecal samples from which both *E. coli* and *Campylobacter* spp. were isolated. Multi-bacterial-antimicrobial resistance was defined, in this study based on the following criteria: 1) any combination of *Campylobacter* spp. and *E. coli* from a sample from which *Campylobacter* spp. that was resistance to at least one of six antimicrobial drugs and from which *E. coli* that were resistant to at least one of 14 antimicrobial drugs were isolated, and 2) that the selected *Campylobacter* spp. and *E. coli* isolates were cumulatively resistant to three or more antimicrobial drugs. Differences between the pigs that had isolates with a combined total of resistance to three or more antimicrobial drugs among *Campylobacter* and *E. coli*, as well as MDR of *Campylobacter* and *E. coli*, were analyzed using a population averaged logistic model as described above.

4.3. Results

Thirty-five antimicrobial-free and 60 conventional farms from 8 Midwestern states were enrolled in this cross-sectional study. The number of years that antimicrobial-free farms had not used antimicrobial drugs ranged from 1 to 14, with a median of 3 years. The mean number of pigs on antimicrobial-free farms was 1262 (range 150-11000; median 800), whereas the mean size for conventional farms was 7909 (range 500-45000; median 4800) ($P<0.001$).

Fifteen fecal samples were collected from late-stage finisher pigs on antimicrobial-free and conventional farms except on one farm where only 12 finisher pigs were available for sampling. A total of 512 *Campylobacter* isolates were isolated

from feces of one or more pigs on 90 of the 95 farms (33 of 35 antimicrobial-free farms and 57 of 60 conventional farms). Of the 512 *Campylobacter* isolates, 464 were available for susceptibility testing; these isolates were obtained from pigs on 30 of the 33 antimicrobial-free farms and 55 of the 57 conventional farms that had *Campylobacter* isolates. On the 95 swine farms, 1,381 (97.1%) *E. coli* isolates were recovered from 1,422 fecal specimens and at least 12 *E. coli* isolates were obtained from all 95 farms (with the exception of one farm that only had 4 *E. coli* isolates from 15 pigs).

4.3.1 Variable description

A total of 38 variables (33 dichotomous and five categorical variables) were used for the initial analyses (see description of variables in Appendix F). Variables regarding the types of antimicrobial drugs used were excluded from this study since antimicrobial-free farms did not use antimicrobial drugs. Explanatory variables were divided into 5 categories: biosecurity, disease history, vaccines used, farm management practices, and medication history. Among vaccines used in finishers, data on six vaccines were dropped because farms reported no usage (PRRS, Swine flu, *Salmonella*, Erysipelas, atrophic rhinitis, and *Escherichia coli* vaccines). Additionally, three disease conditions were excluded due to lack of variability (Circovirus or Post-weaning Multisystemic Wasting Syndrome (PMWS), swine dysentery, and pseudorabies).

Most of the antimicrobial-free farms (91%) were classified as farrow-to-finish farms and the remaining three farms were grower-to-finish farms. Thirty-nine of 60 (65%) conventional farms were farrow-to-finish, 17 were grower-to-finish, three were wean-to-finish, and six were derivations of the above. The majority of the antimicrobial-

free farms (80%) were open (i.e. introduction of purchased replacement pigs was practiced: breeder stock, nursery pigs from off-site farrowing or nursery units, or feeder pigs) whereas (65%) of the conventional farms were open. In addition, 71% antimicrobial-free farms brought in breeding stocks compared to 53% of conventional farms; seven percent of conventional farms brought in nursery pigs whereas none of the antimicrobial-free farms did. Feeder pigs were brought onto 14% of antimicrobial-free farms and 5% of conventional farms. All explanatory variables were measured at the farm level. Explanatory variables were summarized by antimicrobial-free and conventional herd types **(Tables 4.1(a)-4.1(e)).**

4.3.2 Model descriptions

Analysis of each antimicrobial-bacteria combination, MDR, and MBAR to each of the 38 explanatory variables did not reveal common patterns (**see** Appendix G). The final multivariate models for each of the seven antimicrobial-bacteria combinations also had a variety of significant covariates associated with antimicrobial resistance among the seven models (**Tables 4.2 -4.8)**. Herd type was significant in all multivariate models ($P<0.001$). In the multivariable model for *E. coli*-streptomycin, the interaction of swine flu and herd type and the interaction of ulcer to herd type were significant ($P<0.05$); therefore, the interaction terms were included for the *E. coli*-streptomycin multivariable model.

Table 4.1 (a). Summary of herd-level biosecurity variables (risk factors) by antimicrobial-free and conventional swine farms.

Variable name	Farm type	No./total farms	Percentage (95% CI)
vistoronfarm_0	ABF	4/35	11.7 (8.9-14.34)
	Conv	2/60	3.3 (2.16-4.51)
visitoronfarm_1	ABF	24/35	68.2 (64.20-72.20)
	Conv	37/60	61.7 (58.49-64.85)
visitoronfarm_2	ABF	7/35	20.1 (16.67-23.56)
	Conv	21/60	35.0 (31.88-38.12)
toilet_0	ABF	20/35	57.1 (40.29-73.99)
	Conv	18/60	30.0 (18.15-41.85)
toilet_1	ABF	15/35	42.8 (26.0-59.7)
	Conv	42/60	70.0 (58.1-81.8)
exterm	ABF	20/35	8.6 (6.20-11.03)
	Conv	58/60	1.7 (0.80-2.50)
rendering	ABF	10/35	28.6 (13.2-43.9)
	Conv	11/60	18.3 (8.3-28.3)
birdproof	ABF	1/35	2.8 (-2.8-8.5)
	Conv	40/60	66.7 (54.5-78.8)
newlivestock	ABF	23/35	65.7 (49.5-81.9)
	Conv	34/60	56.7 (43.8-69.5)
free_roam	ABF	13/35	37.1 (20.7-53.6)
	Conv	7/60	11.7 (3.4-20.0)
chickens	ABF	12/35	34.3 (18.1-50.4)
	Conv	5/60	8.3 (1.2-15.5)
newlivestock	ABF	19/35	54.3 (37.3-71.2)
	Conv	2/60	20.0 (9.7-30.3)
animal_contact	ABF	13/35	37.1 (20.7-53.6)
	Conv	4/60	6.7 (0.2-13.1)
_acclim_0	ABF	12/35	34.5 (30.40-38.57)
	Conv	31/60	51.7 (48.40-54.44)

Table 4.1 (a). (continued)

Variable name	Farm type	No./total farms	Percentage (95% CI)
_acclim_1	ABF	9/35	25.9 (22.10-29.63)
	Conv	8/60	13.3 (11.11-15.56)
_acclim_2	ABF	14/35	39.6 (35.45-43.86)
	Conv	21/60	35.0 (31.88-38.12)

ABF = Antimicrobial-free farms. Conv = Conventional farms.
CI = Confidence interval.
A key for the variables in this table is presented in appendix F.

Table 4.1 (b). Summary of herd-level disease history variables by antimicrobial-free and conventional swine farms.

Variable name	Farm type	No./total farms	Percentage (95% CI)
actino	ABF	1/35	2.86 (-2.81-8.5)
	Conv	4/60	6.67 (0.22-13.1)
PRRS	ABF	0/35	0
	Conv	24/60	40.0 (27.3-52.7)
swineflu	ABF	5/35	14.3 (2.4-26.2)
	Conv	25/60	41.7 (28.9-54.4)
salm	ABF	4/35	11.4 (0.6-22.3)
	Conv	2/60	3.33 (-1.3-7.97)
Glassers	ABF	2/35	5.7 (-2.1-13.6)
	Conv	9/60	15.0 (5.8-24.2)
myco_pn	ABF	3/35	8.6 (81.9-100.96)
	Conv	23/60	38.3 (25.8-50.9)
rhin	ABF	3/35	8.6 (-0.96-18.1)
	Conv	2/60	3.3 (-1.3-7.97)
hbs	ABF	3/35	8.6 (-0.96-18.1)
	Conv	25/60	41.7 (28.9-54.4)
ili	ABF	7/35	20.0 (6.4-33.6)
	Conv	24/60	40.0 (27.3-52.7)
ulcer	ABF	1/35	2.86 (-2.81-8.5)
	Conv	14/60	23.3 (12.4-34.3)
erysip	ABF	4/35	11.4 (0.6-22.3)
	Conv	5/60	8.33 (1.2-15.5)

ABF = Antimicrobial-free farms. Conv = Conventional farms.
CI = Confidence interval.
A key for the variables in this table is presented in appendix F.

Table 4.1 (c). Summary of vaccine usage at the herd level by antimicrobial-free and conventional swine farms.

Variable name	Farm type	No./total farms	Percentage (95% CI)
vaccine	ABF	6/35	17.1 (4.3-30.0)
	Conv	13/60	21.7 (11.0-32.3)
pseudovx	ABF	4/35	11.4 (0.6-22.3)
	Conv	5/60	8.3 (1.2-15.5)
mycovx	ABF	1/35	2.8 (-2.8-8.5)
	Conv	9/60	15.0 (5.8-24.2)

ABF = Antimicrobial-free farms. Conv = Conventional farms.
CI = Confidence interval.
A key for the variables in this table is presented in appendix F.

Table 4.1 (d). Summary of management practice variables at the herd-level by antimicrobial-free and conventional swine farms.

Variable name	Farm type	No./ total Farms	Percentage (95% CI)
mixfarm	ABF	31/35	88.5 (8.75-14.23)
	Conv	35/60	58.3 (55.11-61.56)
premix	ABF	6/35	17.2 (13.995-20.49)
	Conv	33/60	55.0 (51.75-58.25)
corn	ABF	3/35	8.6 (6.21-11.03)
	Conv	11/60	18.3 (15.80-20.86)
soybean	ABF	11/35	31.0 (27.06-35.01)
	Conv	20/60	33.3 (30.25-36.42)
manurespread	ABF	6/35	17.1 (4.3-29.98)
	Conv	6/60	10.0 (2.2-17.8)
house_1	ABF	3/35	8.6 (6.21-11.03)
	Conv	42/60	61.7 (58.49-64.85)
house_2	ABF	11/35	31.0 (27.06-35.01)
	Conv	9/60	15.0 (12.67-17.34)
house_3	ABF	5/35	14.4 (11.35-17.38)
	Conv	1/60	1.7 (0.83-2.50)
house_4	ABF	14/35	40.2 (36.06-44.44)
	Conv	11/60	18.3 (15.80-20.86)
house_5	ABF	2/35	5.8 (3.75-7.75)
	Conv	2/60	3.3 (2.16-4.51)
flooring_0	ABF	23/35	65.5 (61.43-69.6)
	Conv	56/60	93.3 (91.70-94.97)
flooring_1	ABF	12/35	34.5 (30.40-38.57)
	Conv	4/60	6.7 (3.40-6.43)
floor_0	ABF	32/35	91.4 (88.97-93.79)
	Conv	13/60	21.7 (18.97-24.36)
floor_1	ABF	3/35	8.6 (6.21-11.03)
	Conv	47/60	78.3 (75.64-81.03)

Table 4.1(d). (continued)

Variable name	Farm type	No./ total Farms	Percentage (95% CI)
_Ibedding_0	ABF	3/35	8.6 (6.21-11.03)
	Conv	40/60	66.7 (63.58-69.75)
_Ibedding_1	ABF	25/35	71.8 (67.98-75.70)
	Conv	13/60	21.7 (18.97-24.36)
_Ibedding_2	ABF	7/35	19.5 (16.13-22.95)
	Conv	7/60	11.7 (9.57-13.77)
_Ivent_0	ABF	29/35	82.8 (79.51-86.00)
	Conv	4/60	6.7 (5.03-8.30)
_Ivent_1	ABF	3/35	8.6 (6.21-11.03)
	Conv	28/60	46.7 (43.40-49.90)
_Ivent_2	ABF	3/35	8.6 (6.21-11.03)
	Conv	28/60	46.7 (43.40-49.90)
aiao_0	ABF	12/35	34.5 (30.10-38.67)
	Conv	10/60	16.7 (14.23-19.10)
aiao_1	ABF	23/35	65.5 (61.43-69.60)
	Conv	50/60	83.3 (80.90-85.77)

ABF = Antimicrobial-free farms. Conv = Conventional farms.
CI = Confidence interval
A key for the variables in this table is presented in appendix F.

Table 4.1 (e). Summary of medication usage at the herd level by the number of farms that used these medications and the proportion on antimicrobial-free and conventional swine farms.

Variable name	Farm type	No./total farms	Percentage (95% CI)
dewormer	ABF	22/35	62.8 (46.4-79.3)
	Conv	17/60	28.3 (16.7-40.0)
mangelice	ABF	10/35	28.6 (13.2-44.0)
	Conv	4/60	6.7 (0.2-13.1)
probiotics	ABF	12/35	34.2 (18.1-50.4)
	Conv	1/60	1.7 (-1.6-5.0)

ABF = Antimicrobial-free farms. Conv = Conventional farms.
CI = Confidence interval
A key for the variables in this table is presented in appendix F.

Table 4.2. Multivariable model of herd-level risk factors for azithromycin resistance in *Campylobacter* isolates from finisher pigs on 95 swine farms.

Covariate	B (SE)	OR	95%CI	p-value†	Wald p (df)
htype	2.0 (0.56)	7.5	2.5-22.4	0.00	
mixfarm	-1.4 (0.49)	0.25	0.1-0.6)	0.004	
rhin	-2.7 (0.48)	0.07	0.03-0.17	0.00	
_vent_0	_	_	_	_	_
_vent_1	0.12 (0.58)	1.1	0.36-3.52	0.84	
_vent_2	1.29 (0.46)	3.6	1.48-8.85	0.01	0.00 (2)
_Iacclim_0	_	_	_	_	_
_Iacclim_1	1.81 (0.58)	6.1	1.96-19.24	0.00	
_Iacclim_2	0.86 (0.47)	2.4	0.94-6.01	0.07	0.01 (2)

B = Regression coefficient. SE = Standard error. OR = Odds ratio. CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
*rhinitis and htype interaction caused the model to not converge.
A key for the variables in this table is presented in appendix F.

Table 4.3. Multivariable model of herd-level risk factors for tetracycline resistance in *Campylobacter* isolates from finisher pigs on 95 swine farms.

Covariate	**B (SE)**	**OR**	**95%CI**	**p-value†**
htype	3.45 (0.55)	15.2	5.29-43.89	<0.01
ili	-0.84 (0.34)	0.43	0.22-0.84	0.01
floor	-1.52 (0.48)	0.22	0.08-0.56	0.002
swineflu	-0.73 (0.35)	0.48	0.24-0.96	0.04

B = Regression coefficient. SE = Standard error. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
*no interaction terms were significant.
A key for the variables in this table is presented in appendix F.

Table 4.4. Multivariable model of herd-level risk factors for tetracycline resistance in *E. coli* isolates to from finisher pigs on 95 swine farms.

Covariate	B (SE)	OR	95%CI	p-value†	Wald p (df)
htype	0.71 (0.28)	2	1.18-3.54	0.01	
_Ihouse_2	0.21 (0.42)	1.24	0.55-2.79	0.61	
_Ihouse_3	-0.58 (0.46)	0.56	0.23-1.36	0.20	
_Ihouse_4	0.35 (0.42)	1.42	0.63-3.20	0.40	
_Ihouse_5	1.71 (0.30)	5.53	1.07-28.63	0.04	0.02 (4)
floor	0.77 (0.30)	2.15	1.20-3.86	0.01	
myco_pn	0.88 (0.37)	2.41	1.18-4.93	0.02	

B = Regression coefficient. SE = Standard error. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
*floor probably a confounder for htype.
A key for the variables in this table is presented in appendix F.

Table 4.5. Multivariable model of herd-level risk factors for streptomycin resistance in *E. coli* isolates from finisher pigs on 95 swine farms.

Covariate	B (SE)	OR	95%CI	p-value†	Wald p (df)
htype	0.96 (0.20)	2.6	1.77-3.84	0	
_Ihouse_1					
_Ihouse_2	0.08 (0.23)	1.1	0.69-1.69	0.72	
_Ihouse_3	-0.92 (0.52)	0.4	0.14=1.10	0.08	
_Ihouse_4	-0.13 (0.20)	0.9	0.59-1.31	0.53	
_Ihouse_5	0.67 (0.25)	2	1.20-3.19	0.01	0.00 (4)
flooring	0.89 (0.23)	2.4	1.54-3.83	0.00	
mangelice	0.40 (0.21)	1.5	0.98-2.25	0.06	
ulcer	0.04 (0.28)	1	0.60-1.78	0.90	
free_roam	-0.52 (0.17)	0.6	0.42-0.83	0.00	
Swineflu	0.09 (0.23)	1.1	0.70-1.72	0.69	
flu*htype	-0.71 (0.30)	0.5	0.27-0.89	0.02	
ulcer*htype	-0.72 (0.35)	0.5	0.25-0.96	0.04	

B = Regression coefficient. SE = Standard error. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
A key for the variables in this table is presented in appendix F

Table 4.6. Multivariable model of herd-level risk factors for ampicillin resistance in *E. coli* isolates from finisher pigs on 95 swine farms.

Covariate	**B (SE)**	**OR**	**95%CI**	**p-value†**
htype	0.66 (0.23)	1.9	1.24-3.02	0.004
rhin	-1.71(0.44)	0.2	0.08-0.43	0.000

B = Regression coefficient. SE = Standard error. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
A key for the variables in this table is presented in appendix F.

Table 4.7. Multivariable model of herd-level risk factors for sulfamethoxazole resistance in *E. coli* isolates from finisher pigs on 95 swine farms.

Covariate	B (SE)	OR	95%CI	p-value†	Wald p (df)
htype	0.54 (0.21)	1.7	1.13-2.61	0.01	
size	-0.26 (0.25)	0.8	0.47-1.25	0.29	
_Ihouse_1	-	-	-	_	
_Ihouse_2	-0.01 (0.27)	1.0	0.58-1.67	0.96	
_Ihouse_3	-0.79 (0.41)	0.5	0.20-1.00	0.05	
_Ihouse_4	-0.38 (0.25)	0.7	0.41-1.12	0.13	
_Ihouse_5	0.46 (0.33)	1.6	0.83-3.03	0.17	0.01 (4)
pseudovx	0.58 (0.25)	1.8	1.1-3.0	0.02	

B = Regression coefficient. SE = Standard error. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
A key for the variables in this table is presented in appendix F.

Table 4.8. Multivariable model of herd-level risk factors for chloramphenicol resistance in *E. coli* isolates from finisher pigs on 95 swine farms.

Covariate	B (SE)	OR	95% CI	p-value†	Wald p (df)
htype	0.24 (0.41)	1.3	0.57-2.85	0.56	
chickens	1.12 (0.28)	3.1	1.79-5.32	0.00	
rendering	-1.20 (0.540	0.3	0.10-0.86	0.03	
_Ivent_0	_	_	_	_	
_Ivent_1	1.36 (0.53)	3.9	1.38-11.0	0.01	
_Ivent_2	1.53 (0.51)	4.6	1.70-12.6	0.00	0.01 (2)

B = Regression coefficient. SE = Standard error. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
*chickens and ventilation are confounders of htype
A key for the variables in this table is presented in appendix F.

Herd management practices including type of house, ventilation, bedding, floor type, and flooring were hypothesized to be correlated. Associations were detected based on chi-square associations between each pair of variables listed above. Spearman's Correlation test for independence showed highly significant association between the variables 'bedding' and 'house.' Hence, the variable bedding was excluded from development of the multivariable models.

Herd size was investigated as a potential confounder in all multivariate models; however, adding herd size did not change the overall effect of herd type (conventional and antimicrobial-free) by more than 20% in any of the models except for *E. coli*-sulfamethoxazole and the MDR of *E. coli* (see below); therefore, herd size was included as a confounder in the *E. coli*-sulfamethoxazole model.

Some covariates were associated with antimicrobial resistance in more than one multivariable model given herd type. The 'housing environment' which describes whether a farm used complete confinement (referent), used partial confinement, pasture, hoop barns or other combinations of confinement types, was the variable that was most often associated with AMR of *E. coli* isolates (streptomycin ($P<0.01$), tetracycline ($P<0.02$), and sulfamethoxazole ($P<0.01$)). In the three multivariable models with housing environment as a covariate, other covariates that were significant in the models included floor type, presence of *Mycoplasma pneumonia* (tetracycline); use of pseudorabies vaccine (sulfamethoxazole); in addition, treatment of mange and lice, history of gastric ulcers and swine flu, and allowing free roaming animals on farm (streptomycin) was also present in studies where housing environment was a covariate.

Among three multivariable models (tetracycline, streptomycin, and sulfamethoxazole), pasture was negatively associated with a higher proportion of AMR whereas partial confinement was associated with a higher proportion of AMR, and hoop barns was associated with a higher proportion of tetracycline AMR, but negatively associated with *E. coli*-streptomycin and *E. coli*-sulfamethoxazole AMR.

The flooring variable described whether a farm used dirt, concrete, or some other combination thereof. Use of dirt and other combinations (dirt and concrete, dirt and other type of floor, or concrete and other type of floor) compared to only concrete flooring was associated with streptomycin resistance of *E. coli* isolates. Mixing of feed on the farm as compared to mixing feed offsite was significantly associated with lower azithromycin resistance in *Campylobacter* isolates.

Use of natural or mechanical ventilation on a farm compared to a barn being open to the outside (referent) was positively associated with azithromycin resistance of *Campylobacter* isolates ($P<0.01$) and chloramphenicol resistance of *E. coli* isolates ($P=0.01$). The floor type, slats, weaved or a combination of slats and weaved compared to solid floors was positively associated with tetracycline resistance among *E. coli* isolates (OR=2.15) but negatively associated with tetracycline resistance among *Campylobacter* isolates (OR=0.22).

Biosecurity practices that were significantly associated with resistance prevalence in at least one of the multivariable models were: ‘acclimation,’ ‘allowing free roaming animals on the farm,’ the ‘presence of chickens on the farm,’ ‘allowing rendering trucks on the farm,’ and ‘allowing pig contact with other animals,’ The

acclimation variable (use of mummies, cull animals, sick animals or feces to acclimate replacement animals) was positively associated (OR=2.4) with azithromycin resistance in *Campylobacter* isolates given herd type. Use of vaccines as a method of acclimation (OR=6.1) (referent was acclimation not used) was positively associated among farms that had a higher proportion of azithromycin resistance as compared to farms that did not. Farms with chickens were (OR=3.1) more likely to have chloramphenicol resistance of *E. coli*. Additionally, farms that allow rendering trucks onto farm were less likely to have chloramphenicol resistance given the other variables in the model (OR=0.3).

Accounting for herd type, diseases that were present on the farms within a year of the study and that were significant in one or more multivariable models included rhinitis, ileitis, swine flu, *Mycoplasma pneumonia,* and gastric ulcers. The farm level covariate, 'history of atrophic rhinitis' on a farm, was less likely on farms with a higher proportion of azithromycin resistance of *Campylobacter i*solates (OR=0.07) and ampicillin resistance of *E. coli* isolates (OR=0.2). Tetracycline resistance of *Campylobacter* was less likely on farms with a history of ileitis (OR=0.43) and swine flu (OR=0.48). A history of Mycoplasma pneumonia was positively associated (OR=2.4) with tetracycline resistance of *E. coli*. Both a history of gastric ulcers and swine flu were included as interaction terms with herd type in the multivariable model for streptomycin resistance in *E. coli (P*=0.04 and *P*=0.02, respectively*)*. Use of pseudorabies vaccine was positively associated with sulfamethoxazole resistance in *E. coli* (OR=1.8). In addition, treatment for mange and lice was positively associated with streptomycin resistance in *E. coli* isolates (OR=1.5).

Table 4.9. Multivariable model of herd-level risk factors for multidrug resistance in *Campylobacter* isolates in a study of 95 swine farms. Multidrug resistance was resistance to two or more antimicrobial drugs.

Covariate	B (SE)	OR	95%CI	p-value†
htype	1.6 (0.39)	4.8	2.2-10.4	0.00
hsize*	0.0 (0.00)	1.0	1.0-1.0	0.002
rhin**	-1.6 (0.43)	0.20	0.09-0.47	0.00

B = Regression coefficient. SE = Standard error. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
* herd size added as a confounder
** rhinitis interaction caused the model to not converge
A key for the variables in this table is presented in appendix F.

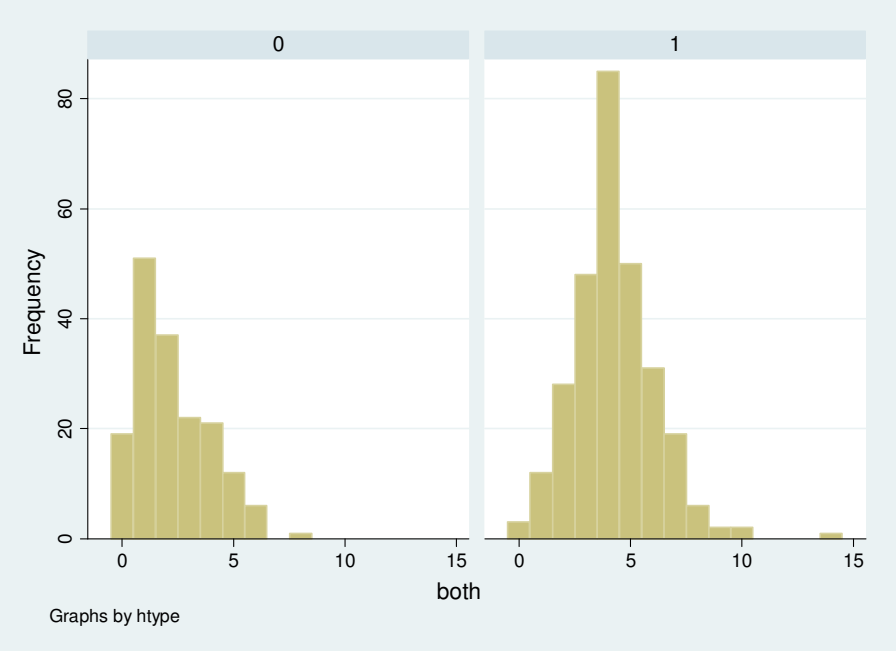
Among 464 *Campylobacter* isolates, MDR was negatively associated with the history of rhinitis on the farm (**Table 4.9**). In addition, adjusted OR were reported to account for herd size which was included in the multivariable model as a confounder. Among 1,381 *E. coli* isolates, MDR was positively associated with the history of salmonellosis (OR=3.6) (**Table 4.10**). MDR was negatively associated with house type (referent was total confinement) ($P<0.01$), history of rhinitis on the farm (OR=0.47), history of swine flu on the farm (OR=0.58), and biosecurity procedures associated with visitors that enter farms (referent was no biosecurity requirements) ($P<0.01$). In addition, herd size was not a confounder in the multivariable model for *E. coli*-MDR. However, an interaction between the history of salmonellosis and herd type was significant and included in the final multivariable model.

Among the 464 pigs that gave rise to a *Campylobacter* isolate, 456 pigs had a *Campylobacter* isolate that was resistant to one or more of 6 antimicrobial drugs and an *E. coli* isolate that was resistant to one or more of 14 antimicrobial drugs (the MBAR model). The median number of resistances per individual pig was 4 with a range of 0-14 (an isolate that was not resistant to any of the antimicrobial drugs was considered pan-susceptible). Among antimicrobial-free farms, there were 169 pigs that had multiple resistances with a median of 2 and a range of 0-8 and among conventional farms, there were 287 pigs with multiple resistances with a median of 4 and a range of 0-14 (**Figure 4.1**). The frequencies of resistance among *Campylobacter* and *E.* coli within one animal were cross-tabulated (**Table 4.11**). Comparison of multiple-resistance among *Campylobacter* and *E. coli* indicated non-significant differences ($P=0.52$):

Table 4.10. Multivariable model of herd-level risk factors for multidrug resistance (MDR) in *E. coli* isolates from finisher pigs on 95 swine farms.

Covariate	B (SE)	OR	95%CI	p-value†	Wald p (df)
htype	0.91 (0.21)	2.5	1.63-3.74	0.00	
_Ihouse_1	_	_	_	_	
_Ihouse_2	0.06 (0.27)	1.1	0.62-1.79	0.83	
_Ihouse_3	-0.82 (0.33)	0.4	0.23-0.85	0.84	
_Ihouse_4	0.14 (0.27)	3.6	1.48-8.85	0.01	
_Ihouse_5	0.08 (0.24)	1.1	0.67-1.75	0.62	0.04 (4)
swineflu	-0.54 (0.22)	0.6	0.38-0.89	0.01	
salm	1.28 (0.20)	3.6	2.42-5.38	0.00	
rhin	-0.75 (0.13)	0.5	0.36-0.61	0.00	
_Ivisitoronfarm_0	_	_	_	_	
_Ivisitoronfarm_1	-0.70 (0.27)	0.5	0.29-0.85	0.01	
_Ivisitoronfarm_2	-0.57 (0.31)	0.6	0.31-1.04	0.07	0.04 (2)
htype*salm	-1.24 (0.46)	0.3	0.12-0.72	0.01	

B = Regression coefficient. SE = Standard error for B. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
*rhinitis and herd-type interaction terms caused the model to not converge.
**Herd-type and *Salmonella* was a significant interaction term.
A key for the variables in this table is presented in appendix F.



0=ABF farms and 1=Conventional farms

Figure 4.1. Frequency of samples with multi-resistance among antimicrobial-free (ABF) and conventional swine farms.

Table 4.11. Frequency of co-resistance among *Campylobacter* and *E. coli* isolates in swine farms.

Campylobacter--	1	2	3	4	5	6	Total
E. coli							
1	22	23	8	15	0	0	68
2	40	41	27	73	0	0	181
3	16	16	12	38	1	0	83
4	12	16	10	27	1	1	67
5	5	14	7	17	0	0	43
6	0	2	0	6	0	0	8
7	0	1	0	2	0	0	3
8	0	0	0	1	0	0	1
9	0	0	1	0	0	0	1
12	0	0	0	1	0	0	1
Total	95	113	65	180	2	1	456

*Pearson Chi2(45) = 43.944; Pr = 0.517

Table 4.12. Multivariable model of herd-level risk factors for multi-bacterial-antimicrobial resistance (MBAR) in *E. coli* and *Campylobacter* isolates from finisher pigs on 95 swine farms.

Covariate	B (SE)	OR	95% CI	p-value†
htype	2.25 (0.31)	9.5	5.20-17.25	0.00
rhin	-0.24 (0.38)	0.8	0.37-1.68	0.54
animal_contact	-0.74 (0.31)	0.5	0.26-0.88	0.02
rhin * htype	-1.71 (0.48)	0.2	0.07-0.47	0.00

B = Regression coefficient. SE = Standard error. OR = Odds ratio.
CI = Confidence interval.
†A value of $P \leq 0.05$ was considered significant.
A key for the variables in this table is presented in appendix F.

The analysis of multiple resistances among pigs with *Campylobacte*r and *E. coli* and the 38 explanatory variables is presented in a table in Appendix G**.** The final multivariable model for MBAR (**Table 4.12.)** included a negative association with animal contact (OR=0.5) and an interaction term between rhinitis and herd type.

4.4. Discussion

The apparent prevalence of antimicrobial resistance has been described in and compared between antimicrobial-free (including organic) and conventional farms for different food animal species: cattle (Sato et al., 2004a; Sato et al., 2005; Halbert et al., 2006; Ray et al., 2006), poultry (Avrain et al., 2003; Luangtongkum et al., 2006; Siemon et al., 2007; Schwaiger et al., 2008), and swine (Docic and Bilkei, 2003; Gebreyes et al., 2005; Bunner et al., 2007; Rollo et al., 2010). In this study, we investigated whether certain herd level practices were associated with antimicrobial resistance in *Campylobacter* and *E. coli* isolated from pigs on antimicrobial-free and conventional swine farms in Midwestern states. The main exposure variable being tested was herd type, i.e., antimicrobial-free versus conventional production practices. Herd type was included in all models.

Previously, the proportions of AMR in swine fecal *E. coli* and *Campylobacter* isolates by herd type were examined using this dataset (Bunner et al., 2007; Rollo et al., 2010). Two antimicrobial drugs (tetracycline and azithromycin) that had a higher proportion of AMR in *Campylobacter* isolates and five antimicrobial drugs (ampicillin,

chloramphenicol, streptomycin, sulfamethoxazole, tetracycline) that had a higher proportion of AMR in *E. coli* in conventional farms as compared to conventional farms were used in the current analysis to investigate if other management practices were associated with the levels of antimicrobial resistance. Only 2 drugs were considered in *Campylobacter* isolates because erythromycin and azithromycin are both macrolides and very similar in their patterns of resistance, and the other antimicrobials had sparse data (see Section 2). Five drugs were selected from the *E. coli* data set because these were the ones that a significant difference between herd type and because resistance was sparse for several of the other antimicrobial drugs. Findings in this study indicate that AMR in *Campylobacter* and *E. coli* isolates of swine was associated with unique herd level risk factors among each antimicrobial-bacteria combination.

In addition, multi-drug resistance of *E. coli* and *Campylobacter* and multi-bacterial-antimicrobial resistance were examined. Multi-bacterial-antimicrobial resistance in this analysis included any combination of *Campylobacter* spp. and *E. coli* from a sample from which *Campylobacter* spp. that was resistance to at least one of six antimicrobial drugs and from which *E. coli* that were resistant to at least one of 14 antimicrobial drugs were isolated. Also, the selected *Campylobacter* spp. and *E. coli* isolates were cumulatively resistant to three or more antimicrobial drugs. Multi-bacterial-antimicrobial resistance was significantly higher on conventional farms compared to antimicrobial-free farms.

Cross-resistance and co-resistance likely contributed to MBAR in this study. Since azithromycin and erythromycin are both macrolides, they are almost completely

cross-resistant. Also, chlortetracycline use in feed has been associated with ampicillin resistance, and the use of tylosin increased the risk of AMR in sulfamethoxazole (Varga et al., 2009). Furthermore, co-selection of chloramphenicol and sulfonamide genes located on plasmids has been described (Bischoff et al., 2002; Travis et al., 2006). One contribution to multidrug resistance is the presence of multidrug efflux pumps. Efflux pumps contribute to the intrinsic resistance of *Campylobacter* spp. to a broad range of structurally unrelated antimicrobial agents (Lin et al., 2002; Payot et al., 2004a; Moore et al., 2006). Another mechanism is transfer of resistance determinants via integrons which integrate resistance genes and transfer them among bacteria. Integrons can be transferred themselves or by either plasmids or transposons. There was a higher frequency of *E. coli* bacterial resistance to ampicillin, streptomycin, sulfonamides, and tetracyclines compared to other antimicrobials in this study as well as swine herds elsewhere (Burch et al., 2008). This study did not examine resistance mechanisms at the molecular level; however, the high frequency of resistance of these antimicrobials was likely related to the co-selection as described above.

A surprising result in this study was the association between use of acclimation and azithromycin resistance of *Campylobacter.* Acclimation is an important management practice that may help build immunity in pigs in isolation before they enter into the main herd. Acclimation includes exposure to manure, cull sows, sick pigs, and is often supplemented with vaccines. About 52% of the conventional farms did not use acclimation compared to one-third of the antimicrobial-free farms. Vaccination is used at different stages of swine production for different purposes. Vaccination of the sow helps

stimulate immunity against *E. coli*, *Clostridium perfringens*, atrophic rhinitis and erysipelas that is passed to piglets (Burch et al., 2008). Growing pigs require vaccination for respiratory diseases. Use of vaccination in herd management is a valuable tool for combating disease which in turn could reduce the likelihood of antimicrobial drug use and AMR. The positive association between the use of vaccines for acclimation and resistance in the azithromycin-*Campylobacter* model may have occurred because only farrow to finish farms use acclimation in nursery pigs, and azithromycin resistance of *Campylobacter* was more prevalent among conventional farrowing to finish farms than antimicrobial-free farms. On the other hand, use of vaccination as a preventive measure (other than as a category of acclimation) was not associated with any AMR of bacteria with the exception of the sulfamethoxazole-*E. coli* model that included usage of pseudorabies vaccine. The negative association between pseudorabies vaccine and sulfamethoxazole resistance of *E. coli* may be because it was an intervening variable in that model or added by chance which will occur when there are a number of variables being examined. A number of studies have been conducted to investigate possible associations between herd level risk factors and bacterial prevalence (e.g. *Salmonella*) (Funk and Gebreyes, 2004; Lo Fo Weng et al., 2004; Bahnson et al., 2006; Zheng et al., 2007; Namata et al., 2009); however, very few studies have been conducted specifically to investigate AMR prevalence and association with herd management factors on food animal farms (with the exception of Schuppers et al., 2005). Most likely, this is because of the added cost of determining the MIC of various antimicrobial drugs as opposed to merely determining the prevalence of bacteria. Furthermore, the association of bacterial

prevalence to farm-level risk factors may be more directly related based on a causal pathway. Some management practices have been developed for the purpose of controlling pathogenic bacteria (Burch et al., 2008). With regards to pathogenic bacteria, the farm level risk factors associated with a history of Salmonellosis could also be related to AMR of *Salmonella;* however, that does not necessarily have to be the case. In addition, management practices that have an impact on pathogen loads most likely will also have an impact on antimicrobial use, which could indirectly influence the prevalence of AMR. Although conventional farms used some antimicrobial drugs, the amounts and types were incomplete and not considered further in this study. This may inadvertently have influenced the associations between AMR and farm level management practices given the wide variety of antimicrobial drugs and purposes for use available on swine farms. Further studies investigating antimicrobial resistance and risk factors thereof should be designed to allow control of bacterial prevalence along with antimicrobial use. This may also help to identify potential confounders.

If predictor variables are highly correlated, the standard errors will be inflated from incorrect variance estimates resulting in unstable regression coefficients (Hosmer and Lemeshow, 2000). We considered the inflated standard errors as a reason to drop a variable (i.e. confinement operation or not). In addition, we selected variables according to the investigation of potential association between independent and dependent variables (Dohoo et al., 2003). In this study, the list of variables was reduced on the basis that some variables may serve as proxies for other variables (multicollinearity) (Agresti, 1996). In those cases, the variable that made most biological sense was chosen,

although this is subjective. Some of the management practices were hypothesized as being related (e.g. floor type, housing, flooring, bedding, and ventilation) so potential correlation was considered. Only one pair of covariates was above the cutoff of 0.7: 'bedding' and 'house'. The variable house described whether pigs were maintained under total confinement, partial confinement, pasture, hoop barn, or a combination of these. Bedding referred to no bedding used compared to straw or corn stalks. Total confinement was expected to be highly associated with 'no bedding used' as was the case in this study (39% of all farms used total confinement housing and no bedding). Therefore, the variable 'bedding' was not considered in the multivariable models. Furthermore, 'house' was significant in three multivariable models for *E. coli* (tetracycline, streptomycin, and sulfamethoxazole). The variable 'house' probably accounts for some of the variation associated with herd size which may explain why herd size was not a confounder in most of the final multivariable models. Other options to reduce multicollinearity include principal components analysis and factor analysis, but those methods cannot determine which individual predictor variables have significant associations with the dependent variable — this was the primary goal of this analysis (Dohoo et al., 1996).

We used stepwise regression as the method for selection of variables to be included in the multivariable models. However, stepwise regression has been criticized because the addition or elimination of a variable may not be based on the causal pathway. In addition, statistical control of confounding can be a problem in stepwise regression. Sometimes irrelevant variables can be selected by chance when there is a large number of predictors (Agresti, 1996), and it is hard to differentiate between an intervening variable and another extraneous variable (Dohoo et al., 1996). Therefore each variable should be verified by comparing the estimated coefficient with the coefficient from the univariate model. In addition, the Wald statistics should be examined, and a smaller model can be compared to the larger by examining any significant changes in the coefficients. This process continues until the most parsimonious model is selected. When there are a number of covariates that are examined for inclusion into a multivariable model, some will be included strictly due to chance. This may explain why only a few variables were included in only one multivariable model in this study, and this should be considered when making inferences based on these results.

We used a population averaged model because of the clustered nature of farm animals. In this study, the outcome was tested at the individual pig level, yet the predictors are at the herd level. If clustering is ignored, the variance in the form of the standard errors will be underestimated. GEE models are marginal models, which mean the expected values for a set of covariates are averaged across the population of clusters. The interpretation is more attractive than a subject specific model where a random effect for each cluster is included in the model (Hardin and Hilbe, 2003). The GEE model uses weighted versions of likelihood equations. The weights are based on the underlying covariance matrix and the shape of the matrix can be selected in the modeling process. The exchangeable matrix was used here which assumes that the correlation between pairs of responses is constant (Hosmer and Lemeshow, 2000).

In conclusion, AMR in *Campylobacter* and *E. coli* isolates from swine fecal samples was associated with a variety of production practices among antimicrobial-free and conventional swine farms. Farm-level intervention studies would be helpful in determining the importance of some of the risk factors identified. Some farmers have chosen to pursue antimicrobial-free farming which likely will reduce AMR in the long run. However, when changing to an antimicrobial-free production system, other changes in production management most likely also will occur. Studies that account for or control these 'other' management changes will be valuable in making decisions whether or not, for example, to change from conventional to antimicrobial-free production. Antimicrobial drug use in food animal production may be needed in order to maintain health and well-being of the animals, but may at the same time increase levels of AMR.

An increase in AMR in products of animal origin may potentially increase the risk of consumers acquiring bacteria of food animal origin which are resistant. Resistant bacteria of food animal origin can be either pathogenic or commensal bacteria that may be able to 'share' resistance determinants with pathogens already in the human intestinal tract. Either way, resistant bacteria of food animal origin may be a source of infections in humans that will be difficult to treat using common antimicrobial drugs. As consumers demand more wholesome food animal products, including low levels of antimicrobial-resistant bacteria (when they are present), it will be beneficial to identify factors, beside ceasing antimicrobial drug use, that can help reduce AMR and the potential transmission to humans through the food chain.

5. CONCLUSIONS

For the past 30 plus years, agricultural animal farming has been oriented toward highly structured processes that often involve farming units that use various applications of antimicrobial drugs. Antimicrobial drugs are used for disease treatment, prevention, and control, and for growth promotion (McEwen and Fedorka-Cray, 2002). In the early 1950s, a beneficial effect on production efficiency led to more trials that tested the use of lower dosages of antimicrobials added to feed (Dibner and Richards, 2005). However, antimicrobial resistance in animal populations followed the introduction of antibiotics (Aarestrup, 2006; Guardabassi and Kruse, 2008). The Swann report (1969) addressed the potential of antimicrobial resistance development, and at that time, the concern was directed towards antimicrobial resistance among human pathogens. As food animal production developed into confinement facilities, feed efficiency as well as disease prevention and control were the primary goals of most production managers. Decisions to use dosages aimed at growth promotion were, in part, based on demands for a more uniform and a less costly product. Furthermore, some antimicrobial drugs were approved for both disease prevention and growth promotion, sometimes at different doses, other times at the same dose (McEwen and Fedorka-Cray, 2002).

Antimicrobial resistance continues to be a topic of concern and in the 1990s, the European Union banned four antimicrobials which were being used as growth promoters and which were considered important in treating human diseases (European Commission, 1999). In Denmark, the overall bulk antibiotic use was decreased by

implementing a removal of antimicrobial growth promoters (AGPs) in livestock production (Danish Integrated Antimicrobial Resistance Monitoring and Research Programme; DANMAP, 2004; Aarestrup et al., 2010). In the U.S., the FDA created a discussion document in 2000 that addressed current issues regarding the use of antimicrobials in food animal production and the effects on human health (FDA, 2000). The premise for removing antimicrobials as growth promoters in the 1990s was that antimicrobial resistance in food animals was directly linked to antimicrobial drug resistance in bacteria that are pathogenic to and transferable to humans (Molbak, 2004). Furthermore, the importance of commensal bacteria's ability to carry resistance genes and transfer them to pathogens at a later time was also addressed in the literature (Sunde et al., 1998). These reports corresponded with a lot of negative publicity that made the consumer take notice. As a result, some countries banned antimicrobial growth promotants in feed, and other methods of food production including organic production were explored and expanded.

When AGPs were banned, morbidity or mortality increased in some production systems. Banning AGP use in weanling pigs resulted in higher incidence of disease which subsequently resulted in an increase usage of therapeutic antibiotics (Aarestrup et al., 2010). Hence, a call was made for more scientifically oriented risk assessments to determine the justification of an AGP ban (Snary et al., 2004). Recently, risk assessments have addressed the use of specific AGPs and potential risk to human health (Cox and Popken, 2004; Hurd et al., 2004). They argue that the uses of tylosin and tilmicosin present a very low risk of human illness due to macrolide-resistant

Campylobacter spp. or *E. faecium* based on a quantitative risk assessment. However, the use of other antimicrobial drugs may pose a risk to human health. It is important not to generalize one study to be representative of all potential relationships of AMR in farm animal production and public health.

In the last decade, consumers have demanded more natural foods, and organic farming has become popular. The perception is that organic foods are safer, and sometimes "better", than products from conventional farms. Organic farming in the US is a system of animal production which, among other things, prohibits use of antimicrobials use as described in more detail in the first section of this dissertation. The NOP standards for organic animal farming specify that the production system cannot involve any use of hormones for growth encouragement or any use of antibiotics (however, vaccines are allowed and sick animals can be treated with antimicrobials if permanently removed from the production system). Additional requirements are also included in the NOP standards. These requirements include the use of organic feed and a certain amount of outdoor exposure (USDA, 2010). The NOP develops, implements, and administers national production, handling, and labeling standards for organic agricultural products. By removing antimicrobials from a production system, the prevalence of antimicrobial resistance (AMR) in organic production systems and hence in the food chain should decrease. However, there is ambiguous scientific evidence to date to support this hypothesis as described in Section one of this dissertation. In addition, converting to organic production is more difficult than it sounds. For example, if one of the larger swine producers made the decision to change to organic production, then an

increase in demand for organic food crops would exceed the supply (Baker, 2006). Instead, some producers have designated their production as antimicrobial-free or reduced antimicrobials. This production system is less stringent than organic farming and in general only requires a cessation of antimicrobial use, although other management requirements may be imposed as well.

The term antimicrobial resistance can be described as a microbiological change in a subpopulation of bacteria that in turn leads to new populations that are resistant to antimicrobials (Harrison and Lederberg, 1998; Andersson, 2003). The new populations of bacteria have change(s) in one or more genes (i.e. mutation) or contain plasmids or other mobile genetic elements, all of which render the bacteria resistant to one or more antimicrobial drugs. Resistance means that an increase in the concentration of an antimicrobial drug is required to inhibit growth of or kill the organism. In animals or humans, these increases in antimicrobial concentration in target tissues are not achievable, sometimes referred to as 'clinical resistance.' This selection for resistant determinants that may propagate and then be disseminated in bacterial populations in animals results in higher proportions of resistant bacteria. Movement of resistant determinants between bacteria can occur on mobile genetic elements such as plasmids and transposons (Baquero and Canton, 2009). In general, a gradual increase in the proportion of resistant bacteria occurs after prolonged use of an antimicrobial in a population, due to selective and evolutionary events accruing over time. And even some undetectable changes below breakpoints are noteworthy as a first step towards clinical resistance (Phillips et al., 2004). With the cessation of antimicrobial use in a population

of animals (such as might occur with organic farming), one would expect a decrease and possibly the elimination of resistant bacteria. One way to examine the trends of antimicrobial resistance in farming environments is to compare animal populations that utilize antimicrobial drugs for growth promotion, prevention, control, or treatment to populations that avoid any antimicrobial usage. This project examined these two exposures (no antimicrobial usage and conventional use of antimicrobials in swine farms).

The initial objective of this research project was to critically review the literature for studies in which levels and patterns of bacterial antimicrobial resistance were compared in organic versus conventional farming systems. Specifically, the methodology for conducting a systematic review was used to evaluate and appraise studies reporting a difference in prevalence of AMR between the two management schemes. The hypothesis was that the cessation of antimicrobial use would result in fewer isolates that exhibited antimicrobial resistance. In reviewing the literature we found that there were significant and systematic methodological factors in these studies that limit their usefulness for reaching comparative conclusions about levels of AMR bacteria in organic versus conventional farms. The main focus of the critical review hence shifted to issues that can make generalizations from these studies unreliable and that can cause difficulties in comparison and summation across studies. Some of the problems had to do with the internal validity of the studies, while others were related to their generalizability and to the possibility of making cross-study comparisons. We identified seven specific problem areas. First, we identified that most of the current

literature included studies with inadequate sample sizes. In addition, there was a significant variation in how subjects were sampled on the farm and how bacterial isolates were chosen for antimicrobial susceptibility testing. The predominant method of choosing farms to participate in the study was inconsistent, and nonrandomized selection methods were always used. In addition, the definition of organic and antimicrobial-free farms varied significantly between studies so that, in other words, the exposure (antimicrobial use) was not comparable between studies. The fifth problem area was the inconsistency in methods used to determine and report antimicrobial susceptibility which varied between country and between which bacterial species was studied. Another microbiological problem was the inherent microbiological limitations and potential bias that may occur if similar isolation techniques are not used between studies. The final area of discussion was the fact that many studies used inappropriate statistical models for the structure and nature of the data. The three specific statistical problems that we identified were a) not accounting for the hierarchical structure of the data; b) the failure to account for the censored nature of the MIC distribution; and c) the issue of discrete outcomes when comparing MIC distributions and the inadequacy of the Cox regression model.

These major issues limit the cross-summation or inference from these types of studies. Although we found that more studies suggested a greater prevalence of antimicrobial resistance on conventional farms, some studies suggested the opposite, and the prospect of reaching any definitive conclusion on the basis of the existing literature seems extremely unlikely.

In the second section of this dissertation, the objective of the study was to identify and compare the apparent prevalence of *Campylobacter* spp. and the apparent prevalence and patterns of AMR for fecal *Campylobacter* spp. isolated from pigs reared under antimicrobial-free and conventional production methods. This project was designed as a cross-sectional study of antimicrobial-free swine farms selected from a list of 2 co-ops. Thirty-five farms agreed to participate in the study which involved visiting each farm once to collect 15 fecal samples from finishers and the administration of a questionnaire for farm managers. Once the 35 farms were selected, conventional farms were selected that were close in proximity to each antimicrobial-free farm. Herd size was also considered but in most cases, conventional farms were larger than antimicrobial-free farms therefore identifying small conventional farms was unrealistic. Fifteen fecal samples from 15 finishers were collected from each farm with the exception of one farm where only 12 samples were collected. The study was conducted in 2002-2003. Healthy finishers were selected because there would be less variation in present treatments and they are closer to slaughter. Farms from seven states were included in this study **(Table 5.1**).

Three bacterial types were selected, *Salmonella*, *Campylobacter*, and *E. coli,* but there were insufficient numbers of *Salmonella* isolates for a statistical analysis. *Campylobacter* spp. are important zoonotic pathogens found in pigs and which may carry resistance determinants (Pezzotti et al., 2003; Rollo et al., 2010). Furthermore, illness caused by the exposure to *Campylobacter* in food can be difficult to treat if resistant strains are present (Helms et al., 2005). *E. coli* are present in the gastrointestinal

Table 5.1. Ninety-five farms from 8 Midwestern states were included in a study of *Campylobacter* prevalence and antimicrobial susceptibility.

	ABF		Conventional		Both		
State	**No. of farms**	**No. of pigs**	**No. of farms**	**No. of pigs**	**Total farms**	**Total Pigs**	**Mean Farm size**
Iowa	20	297	17	255	37	552	2,067
Illinois	7	105	8	120	15	225	3,123
Indiana	0	0	5	75	5	75	12,900
Michigan	0	0	21	315	21	315	13,500
Minnesota	2	30	6	90	8	120	2,988
Nebraska	5	75	1	15	6	90	1,508
Ohio	0	0	2	30	2	30	10,050
Wisconsin	1	15	0	0	1	15	300
Total	35	522	60	900	95	1422	

No. = Number, ABF= antimicrobial-fr**ee**

tract of most warm-blooded animals as a commensal bacterium (Hartl and Dykhuizen, 1984). *E. coli* are also present in the environment and can serve as a reservoir for resistance genes that can be transferred to pathogenic bacteria (Sunde et al., 1998; Windfield and Groisman, 2003;Anderson and Sobsey, 2006). However, the actual transfer of resistant genes from commensal bacteria to pathogenic bacteria has not been thoroughly investigated *in vivo* (Mathew et al., 2007). One theory suggests that the exposure of commensal bacteria to various antimicrobials can lead to an increase in prevalence of genes that are associated with resistance by plasmids, integrons, and transposons (Lees et al., 2008).

In the study of antimicrobial resistance in *Campylobacter* spp. and *E. coli* presented in this dissertation, the microbiological methods used for isolation, identification of bacterial isolates and their resistance patterns included several steps. First, the bacteria were isolated from the fecal samples based on current microbiological methods (see Section 2). Second, the antimicrobial susceptibility was determined. For *Campylobacter* a panel of six antimicrobial drugs was used, and for *E. coli*, a panel of 14 antimicrobial drugs was used (Bunner et al., 2007).

The apparent prevalence of *Campylobacter* among finishers was approximately 33% and the prevalence was independent of herd size and production system (ABF vs. conventional). The highest apparent prevalence of AMR was to erythromycin, azithromycin, and tetracycline. Both macrolides (erythromycin and azithromycin) had similar distributions and there were about 70% more resistant isolates among conventional farms. Tetracycline resistance was evident on all farms and although there

was a significant higher proportion of AMR on conventional farms, the difference between the two production systems was less than the macrolides. One interesting result in this study was an apparent dose-response effect for the duration of antimicrobial-free production.

There was an association with the length of time a farm was free from antimicrobial drug use and the reduced proportion of bacterial antimicrobial resistance. In other words, the more years that a farm was managed without antimicrobial drug use, the less antimicrobial resistance of *Campylobacter* spp. was present on the farm. The apparent dose-response effect was most apparent for the macrolides (see Figure 3.1). Tetracycline resistance had a threshold for decline at approximately 3 years. In other words tetracycline resistance did not decline until a farm was antimicrobial free for 3 or more years. The large variety of mechanisms of tetracycline resistance (see Figure 3.2) among *Campylobacter* spp. isolates may explain why there was only a 40% decrease in tetracycline resistance on farms that were antimicrobial free for $\geq$ 6 years, compared with findings on conventional farms; in contrast, an 80% decrease in erythromycin resistance and an 83% decrease in azithromycin resistance was detected between those farm types. This is relevant because bacteria resistance of tetracycline on the farm will most likely be difficult to eliminate or reduce significantly compared to other antimicrobials.

In the *Campylobacter* study described in section two, decreased AMR to erythromycin, azithromycin, and tetracycline was observed on antimicrobial-free farms. However, this was a cross-sectional study so the rate of decrease in resistance could not

be quantified. Furthermore, one inherent assumption was that both types of farms had the same amount of AMR prior to the change in production to organic practices. Another limitation was that when farms changed to organic production, changes in risk factors most likely would have occurred. The results of this study are valuable for generating hypotheses and the prospect of a longitudinal study which could further characterize potential dose responses that may be associated with the cessation of antimicrobial drug use would be intriguing.

A number of descriptive studies have reported various proportions of antimicrobial resistance among *Campylobacter* spp., *Salmonella*, and *E. coli* in animal populations (Mathew et al., 2001; Sato et al., 2004a; Gebreyes et al., 2005; Englen et al., 2007). The measure of susceptibility was presented as minimum inhibitory concentrations which were then dichotomized into two categories (susceptible and resistant) based on breakpoints for each antimicrobial drug. Using microbiological breakpoints to dichotomize bacteria into susceptible and resistant categories may limit researchers' ability to compare susceptibility among bacteria isolated from animals reared under different production systems. This is particularly troublesome when few or none of the bacteria have MIC values above the breakpoint (i.e. classified as resistant). An alternative to comparing proportions is to compare the distribution of MIC values in two or more populations. The frequency distribution of MICs in a group of bacterial isolates will vary based on the bacteria, the antimicrobial drug, and the proportion of isolates that have unique MICs. The distribution can be right or left censored and often there is a spike in the highest MIC category. In addition, each test to determine MIC

(e.g. Etest® or microbroth dilution) differs in the number of categories available for measuring growth inhibition. Comparing MIC distributions allows the researcher to account for the variation and the range of MICs in a population. Another characteristic of a MIC distribution is that the distribution is not considered continuous; rather, there are a set number of categories. The number of categories differs based on the susceptibility testing method. From a statistical point of view, this is referred to as interval censoring. These inherent characteristics of MIC distributions make the statistical analysis a challenge and addressing this issue was the objective of Section 3.

The objective of the second study reported in Section 3 was to introduce a statistical model that accounted for the inherent characteristics of an MIC distribution including censoring of the data and discrete intervals. The MIC distribution of *E. coli* and *Campylobacter* isolates were compared between antimicrobial-free and conventional farms. We described a discrete time survival analysis model and its suitability for analysis of MIC data. To our knowledge, this model has not been considered and derived in detail for use in comparing MIC distributions between populations previously. Section 3 considers the possibility of using this model. Derivation of this model for this type of data required a transformation of the data from a subject-period data into subject-period time data, a longitudinal progression. Each isolate was represented in multiple lines accounting for time or the lowest concentration of the test to the point where growth inhibition occurred. A hazard function describes the conditional probability of event occurrence at time (t) given it has not occurred up to time (t) (Dohoo et al., 2003). In addition, other assumptions inherent to survival analysis models were also addressed.

Using the DTSA framework, there was a significant difference in the distributions for all six antimicrobial-bacterial combinations tested among antimicrobial-free and conventional farms, whereas when comparing the proportion of resistant isolates, there was no significant difference for two out of six antimicrobial drugs (see Section two). The DTSA model is popular in the social sciences because it allows outcome measures to be grouped into discrete outcomes which are common in some research settings. Although data transformation was cumbersome, this model could provide a framework for assessing subtle differences between two populations when MIC distributions are the outcome. One limitation of this model derivation was the failure to fully incorporate accountability for clustering among isolates within farms. We recommend a more extensive statistical analysis that could incorporate a shared frailty into the model (Rabe-Hesketh and Skrondal, 2005). We recommend that this model be considered when the objective of the study is to determine if there are differences between MIC distributions. The use of a DTSA could potentially be considered when examining the usefulness of an intervention. In a longitudinal context, a population of bacteria could be monitored over time to see if a proposed intervention caused a very subtle change in the MIC frequency distribution. In addition, simulation modeling may shed light on what the actual threshold would be to see an actual difference between two populations.

In conclusion, we have considered MIC data in a unique fashion by introducing a DTSA model, deriving this model, and applying it in a setting that compares bacterial antimicrobial resistance among two production methods. The model seems to

appropriately match the inherent characteristics of the data. Using a DTSA model to compare population MICs is intriguing. The goal of statisticians is to select a statistical model that accurately reflects the nature of the data and the DTSA is the closest model available for this type of data. Other statistical considerations that should be accounted for in studies incorporating farm populations, are accounting for the hierarchical nature of the data.

The objective of the final project reported in this dissertation (Section 4) was to identify the association between farm management practices (other than herd type) and antimicrobial resistance. Very few studies have addressed this issue. Risk factors including management practices such as biosecurity measures, disease prevention, and history of certain diseases have been associated with pathogen apparent prevalence on the farm (Funk and Gebreyes, 2004; Zheng et al., 2007). The association of these practices with a higher proportion of bacterial antimicrobial resistance is lacking. There are several possible reasons for this research gap. First, the association of bacterial prevalence with farm-level risk factors may be more directly related based on a causal pathway. For example, changes of management practices may directly impact pathogen loads which would indirectly impact bacterial antimicrobial resistance. Secondly, the additional cost of conducting antimicrobial resistance testing in a study may be prohibitive. This question may be better addressed in a controlled environment where interventions can be measured and accounted for in the study. However, this project serves the purpose of asking the question in a broad context and associations here may serve as new hypotheses in a more controlled environment.

This study was conducted by collecting herd level risk factors with the use of a questionnaire as described in Section 4. A questionnaire was administered to all participating farm managers to capture farm practices such as biosecurity measures, vaccine usage, and use of preventive measures, and antimicrobials used, as well as disease history. The questionnaire included sections for nursery pigs, sows, gilts, and boars. However, only data collected specifically on finishers was used in the study described here. The questionnaire format was based on data collected for NARMS (CDC, 2003). The goal of this study was to identify potential herd-level risk factors associated with AMR among *Campylobacter* and *E. coli* in pigs from antimicrobial-free and conventional swine farms in the Midwest. Farm-level risk factors were analyzed using multivariable models (representing each antimicrobial and bacteria combination) where the dichotomous outcome represented each individual bacterial isolate as susceptible or resistant based on a predetermined breakpoint. Findings in this study indicate that the prevalence of isolates with AMR in the *Campylobacter* and *E. coli* isolates of swine were associated with unique herd-level risk factors among each antimicrobial-bacteria combination. This emphasizes the complexity of antimicrobial susceptibility on the farm among commensal bacteria. However, the results are important for generating new hypothesizes and considerations when designing controlled studies in the future.

Antimicrobial resistance in agricultural systems is an ongoing concern to both human and animal health (Molbak, 2004; Mathew et al., 2007). Both commensal and pathogenic bacteria obtained from swine farms, including *Campylobacter* spp. and *E.*

coli (Rollo et al., 2010, Taylor et al., 2009; Bunner et al., 2007; Dunlop et al., 1998), may be resistant to a large range of antimicrobials. In many cases, bacterial resistant strains are associated with the type of antimicrobial used on the farm (Harada et al., 2008; Rosengren et al., 2009; Varga et al., 2009); however, there are resistant clones to antimicrobial drugs for which there is no history of farm use (Thakur and Gebreyes, 2005). For example, bacteria that are resistant to fluoroquinolones are apparent on some poultry farms that have never used drugs in this group (Taylor et al., 2009). In addition, in countries in which some antimicrobials are banned, bacterial antimicrobial resistance is still present (Bischoff et al., 2002; Harada et al., 2006). Furthermore, on antimicrobial-free farms, resistance is present although at lower proportions (Rollo et al., 2010; Bunner et al., 2007; Halbert et al., 2007; Sato et al., 2004a; Luangtongkum et al., 2006). A number of studies have addressed mechanisms promoting AMR persistence, and the complex interaction between antimicrobials and bacterial species further complicates the issue (Andersson, 2003).

There are several strategies to reduce AMR in farming environments in addition to converting to an antimicrobial-free farming system. First of all, producers could reduce the quantity of antimicrobial drugs used by considering the following changes. Producers should attempt to reduce disease; for example, there are farms that are certified free of diseases such as *Mycoplasma hyopneumoniae* (Baker, 2006). Vaccine development and usage could also be used for *Salmonella*, *E. coli*, and other diseases (McEwen and Fedorka-Cray, 2002). Some recent changes in production have increased weaning age, which appears to reduce antimicrobial use in young pigs and helps with

maternal immunity. Biosecurity is another management tool used for disease prevention that would mitigate some AMR that could potentially be spread in the environment (by people, other animals, or wildlife). Applying some of these practices may directly influence the bacterial prevalence for pathogens such as *Salmonella,* but indirectly could be related to the prevalence of AMR on the farm. Further strategies that mitigate resistance selection or persistence in both animal and human populations are warranted.

Furthermore, farm level practices may be associated with different proportions of bacterial antimicrobial resistance, and this dissertation explores this possibility. Based on these multivariable models, some new hypotheses should be explored further. Finally, based on the limitations of the current published literature on this subject, we recommend the following. Sampling variation and variance has been explored by several researchers without a clear cut conclusion on the best strategies for sampling farms. This subject should be addressed within each prospective project. Furthermore, the research community should address products and management strategies that are allowed on organic farms, that is, clarify and unify the meaning of 'organic' farms. Breakpoints need to be clarified between countries and for all species of bacteria in animals and when changes occur in breakpoint designations, these should be specified in the literature. The CLSI has improved the standards for antimicrobial susceptibility methods since the early 2000s, but researchers must realize this is a dynamic process and frequently verify any changes in the standards on an annual basis.

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APPENDIX A

DATABASE SEARCH STRATEGY

A. Database: FSTA, BIOSIS Previews, AGRIS
Search Strategy:
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1 (antimicrobial$ or antibiotic$).mp. [mp=ti, ab, ao, ea, fa, sa, sh, ec, ei, fc, fi, fm, ie, lc, oi, si, sm, bc, bo, bt, cb, cc, ds, ge, gn, mc, mi, mq, or, ps, sq, st, tm, tn, hw] (259026)
2 limit 1 to english language (205670)
3 limit 2 to yr="1985 -Current" (182780)
4 (resistance$ or susceptible$ or minimum inhibitory concentration$ or mic$).mp. [mp=ti, ab, ao, ea, fa, sa, sh, ec, ei, fc, fi, fm, ie, lc, oi, si, sm, bc, bo, bt, cb, cc, ds, ge, gn, mc, mi, mq, or, ps, sq, st, tm, tn, hw] (5537098)
5 limit 4 to english language (4381534)
6 limit 5 to yr="1985 -Current" (3769993)
7 (organic$ or antibiotic-free$ or antibiotic free$ or antimicrobial-free$ or antimicrobial-free$).mp. [mp=ti, ab, ao, ea, fa, sa, sh, ec, ei, fc, fi, fm, ie, lc, oi, si, sm, bc, bo, bt, cb, cc, ds, ge, gn, mc, mi, mq, or, ps, sq, st, tm, tn, hw] (387916)
8 limit 7 to english language (302296)
9 limit 8 to yr="1985 -Current" (278720)
10 (swine or pig* or porcine or sow or boar or finisher or cattle or cow$ or heifer$ or dairy or poultry$ or layer$ or hen$ or chicken$).mp. [mp=ti, ab, ao, ea, fa, sa, sh, ec, ei, fc, fi, fm, ie, lc, oi, si, sm, bc, bo, bt, cb, cc, ds, ge, gn, mc, mi, mq, or, ps, sq, st, tm, tn, hw] (1397901)
11 limit 10 to english language (1038831)
12 limit 11 to yr="1985 -Current" (892210)

B. Database: CAB Abstracts <1910 to 2010 Week 26>
Search Strategy:
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1 (antimicrobial$ or antibiotic$).mp. [mp=abstract, title, original title, broad terms, heading words] (99422)
2 limit 1 to (english language and yr="1985 -Current") (48791)
3 (resistance$ or susceptible$ or minimum inhibitory concentration$ or mic$).mp. [mp=abstract, title, original title, broad terms, heading words] (1362705)
4 limit 3 to (english language and yr="1985 -Current") (722423)
5 (organic$ or antibiotic-free$ or antibiotic free$ or antimicrobial-free$ or antimicrobial-free$).mp. [mp=abstract, title, original title, broad terms, heading words] (214556)
6 limit 5 to (english language and yr="1985 -Current") (121885)
7 (swine or pig* or porcine or sow or boar or finisher or cattle or cow$ or heifer$ or dairy or poultry$ or layer$ or hen$ or chicken$).mp. [mp=abstract, title, original title, broad terms, heading words] (1320203)
8 limit 7 to (english language and yr="1985 -Current") (465989)

APPENDIX B

SUMMARY OF 25 STUDIES COMPARING PREVALENCE OF RESISTANT ISOLATES IN ANTIMICROBIAL-FREE AND CONVENTIONAL FARMS

DAIRY/MILK

Reference	Study location	Sample years	Sample types	Bacterium
Bennedsgaard et al., 2006	Denmark	2000–2003	quarter milk samples on farm	*Staphylococcus aureus*
Bombyk et al., 2008	USA (MN)	2004–2005	composite milk samples	*S. aureus*
Cho et al., 2006	USA (MN)	2001–2002	fecal samples	*E. coli* 0157: H7
Cho et al., 2007	USA (MN)	2001–2002	fecal samples	Shiga toxin-producing *E. coli*
Garmo et al., 2010	Norway	2006–2007	quarter milk samples on farm	*S. aureus* coagulase-negative *Staphylococcus*
Halbert et al., 2006	USA (MI, MN, WI, NY)	2000–2001	fecal samples on farms	*Campylobacter* spp.
Pol and Ruegg, 2007	USA (WI)	not specified	quarter milk samples on farm	*S. aureus* coagulase-negative *Staphylococcus* *Streptococcus* spp.

DAIRY/MILK				
Ray et al., 2006	USA (NY, MI, MN, WI)	2000–2001	fecal samples on farms	*Salmonella*
Reinstein et al., 2009	USA (KS)	not specified	not specified	*E. coli* 0157: H7
Roesch et al., 2006	Bern, Switzerland	2003–2004	quarter milk samples on farm	*S. aureus* and coagulase-negative *Staphylococcus* *Streptococcus* spp. (S*trep uberis* and S*trep dysgalactiae*)
Sato et al., 2004a	USA (WI)	2000 and 2001	fecal samples on farms	*Campylobacter* spp.
Sato et al., 2004b	USA (WI) and Denmark	2000	bulk tank milk samples on farm	*S. aureus*
Sato et al., 2005	USA (WI)	2000 and 2001	fecal samples on farms	*E. coli*

POULTRY/CHICKEN				
Reference	**Study location**	**Sample years**	**Sample types**	**Bacterium**
Heuer et al., 2001	Denmark	1998–2000	abattoir	*Campylobacter* spp.
Hoogenboom et al., 2008	The Netherlands	2003 and 2005	fecal samples on farm	*E. coli* 0157: H7 *E. faecium* *Campylobacter* spp.
Luangtongkum et al., 2006	USA (OH)	2000–2002	gastrointestinal tracts at slaughter	*Campylobacter* spp.
Schwaiger et al., 2008	Bavaria, Germany	2004–2005	cloacal swabs on farm	*Campylobacter* spp. *E. coli*
Schwaiger et al., 2009	Bavaria, Germany	2004–2005	cloacal swabs on farm	*Listeria* *Enterococcus* spp.
Siemon et al., 2007	USA (WI, NC, VI, SC)	2004–2005	fecal samples prior to slaughter	*Salmonella*

SWINE/PORK				
Reference	**Study location**	**Sample years**	**Sample types**	**Bacterium**
Bunner et al., 2007	USA (IO,IL, IN, MN, NE, OH, WI)	2002–2003	fecal samples on farms	*E. coli*
Docic and Bilkei, 2003	Hungary, Romania, Serbia	2001	fecal samples on farms	*E. coli*
Gebreyes et al., 2006	USA (NC)	2002–2004	fecal samples from extensive and intensive systems	*Salmonella*
Hoogenboom et al., 2008	The Netherlands	2003 and 2005	fecal samples on farms	*E. coli* 0157: H7 *E. faecium* *Campylobacter* spp.
Mathew et al., 2001	USA (IO, NJ, KY, TN, IN)	prior to 2000	fecal samples on farms	*E. coli* *Salmonella*
Nulsen et al., 2008	New Zealand	2001	fecal samples on farms	*E. coli* *Enterococcus* spp.
Thakur and Gebreyes, 2005	USA (NC)	2002–2004	fecal samples from extensive and intensive systems and three stages of slaughter	*Campylobacter coli*

APPENDIX C

PCR amplicon size and primers used for identification of *Campylobacter* spp. by use of an m-PCR assay.

Target gene	PCR amplicon size (base pairs)	Primer	Sequence (5'-3')
C. jejuni 23S rRNA	650	23SF	TATACCGGTAAGGAGTGCTGGAG
		23SR	ATCAATTAACCTTCGAGCACCG
C. jejuni hipO	323	CJF	ACTTCTTTATTGCTTGCTGC
		CJR	GCCACAACAAGTAAAGAAGC
C. coli glyA	126	CCF	GTAAAACCAAAGCTTATCGTG
		CCR	TCCAGCAATGTGTGCAATG
C. lari glyA	251	CLF	TAGAGAGATAGCAAAAGAGA
		CLR	TACACATAATAATCCCACCC

APPENDIX D

Life table describing the number of *Campylobacter* spp. isolates in each MIC category for azithromycin in a sample of 464 isolates from conventional (*n*=290) and antimicrobial-free (*n*=174) production systems

			Number			Proportion of	
MIC Value (ug/mL)	**MIC category**	**Interval**	**Isolates at the beginning of the MIC scale (*n* at risk)**	**Isolates with an MIC in the interval (*n* events)**	**Censored isolates an the end of the MIC scale (*n* censored)**	**Susceptible isolates whose growth becomes inhibited by AB (hazard function)**	**All isolates that are still susceptible (survivor function)**
Antimicrobial Free Farms							
0.016	1	1, 2					1.0000
0.03	2	2, 3					1.0000
0.064	3	3, 4	174	1	0	0.0058	0.9943
0.125	4	4, 5	173	34	0	0.2179	0.7989
0.25	5	5, 6	139	39	0	0.3264	0.5747
0.5	6	6, 7	100	38	0	0.4691	0.3563
1	7	7, 8	62	27	0	0.5567	0.2011
2	8	8, 9	35	2	0	0.0588	0.1897
4	9	9; 10				-	-
8	10	10; 11				-	-
16	11	11; 12				-	-
32	12	12; 13				-	-
64	13	13; 14				-	-
128	14	14; 15				-	-
256	15	≥15	33	33	33	1	0

MIC Value (ug/mL)	MIC category	Interval	Isolates at the beginning of the MIC scale (*n* at risk)	Isolates with an MIC in the interval (*n* events)	Censored isolates an the end of the MIC scale (*n* censored)	Susceptible isolates whose growth becomes inhibited by AB (hazard function)	All isolates that are still susceptible (survivor function)
Conventional Farms							
0.016	1	1, 2				-	1.000
0.03	2	2, 3				-	1.000
0.064	3	3, 4	290	4	0	0.0139	0.9862
0.125	4	4, 5	286	7	0	0.0248	0.9621
0.25	5	5, 6	279	39	0	0.1503	0.8276
0.5	6	6, 7	240	31	0	0.1381	0.7207
1	7	7, 8	209	9	0	0.044	0.6897
2	8	8, 9	200	2	0	0.0101	0.6828
4	9	9; 10				-	-
8	10	10; 11				-	-
16	11	11; 12				-	-
32	12	12; 13				-	-
64	13	13; 14				-	-
128	14	14; 15				-	-
256	15	≥15	198	198	198	1	0

Life table describing the number of *Campylobacter* spp. isolates in each MIC category for tetracycline in a sample of 464 isolates from conventional (*n*=290) and antimicrobial-free (*n*=174) production systems

			Number			Proportion of	
MIC Value (ug/mL)	**MIC category**	**Interval**	**Isolates at the beginning of the MIC scale (*n* at risk)**	**Isolates with an MIC in the interval (*n* events)**	**Censored isolates an the end of the MIC scale (*n* censored)**	**Susceptible isolates whose growth becomes inhibited by AB (hazard function)**	**All isolates that are still susceptible (survivor function)**
Antimicrobial Free Farms							
0.016	1	1; 2	174	0		-	1.000
0.03	2	2; 3	174	1		0.0057	0.9943
0.064	3	3, 4	173	2	0	0.0116	0.9828
0.125	4	4, 5	171	12	0	0.0702	0.9138
0.25	5	5, 6	159	18	0	0.1132	0.8103
0.5	6	6, 7	141	26	0	0.1844	0.6609
1	7	7, 8	115	8	0	0.0696	0.6149
2	8	8, 9	107	6	0	0.0561	0.5805
4	9	9; 10	101	7		0.0693	0.5402
8	10	10; 11	94	9		0.0957	0.4885
16	11	11; 12	85	8		0.0941	0.4425
32	12	12; 13	77	19		0.2468	0.3333
64	13	13; 14	58	15		0.2586	0.2471
128	14	14; 15	43	13		0.3023	0.1724
256	15	≥15	30	30	30	1	0.1724

MIC Value (ug/mL)	MIC category	Interval	Isolates at the beginning of the MIC scale (*n* at risk)	Isolates with an MIC in the interval (*n* events)	Censored isolates an the end of the MIC scale (*n* censored)	Susceptible isolates whose growth becomes inhibited by AB (hazard function)	All isolates that are still susceptible (survivor function)
Conventional Farms							
0.016	1	1; 2	290	0		-	
0.03	2	2; 3	290	0		-	
0.064	3	3, 4	290	1	0	0.0034	0.9966
0.125	4	4, 5	289	3	0	0.0104	0.9862
0.25	5	5, 6	286	2	0	0.0070	0.9793
0.5	6	6, 7	284	7	0	0.0246	0.9552
1	7	7, 8	277	7	0	0.0253	0.9310
2	8	8, 9	270	16	0	0.0593	0.8759
4	9	9; 10	254	19		0.0748	0.8103
8	10	10; 11	235	19		0.0809	0.7448
16	11	11; 12	216	21		0.0972	0.6724
32	12	12; 13	195	40		0.2051	0.5345
64	13	13; 14	155	29		0.1871	0.4345
128	14	14; 15	126	27		0.2143	0.3414
256	15	≥15	99	99	99	1	0.3414

Life table describing the number of *Campylobacter* spp. isolates in each MIC category for gentamicin in a sample of 464 isolates from conventional (*n*=290) and antimicrobial-free (*n*=174) production systems

			Number			Proportion of	
MIC Value (ug/mL)	**MIC category**	**Interval**	**Isolates at the beginning of the MIC scale (*n* at risk)**	**Isolates with an MIC in the interval (*n* events)**	**Censored isolates an the end of the MIC scale (*n* censored)**	**Susceptible isolates whose growth becomes inhibited by AB (hazard function)**	**All isolates that are still susceptible (survivor function)**
Antimicrobial Free Farms							
0.016	1	1; 2				0	
0.03	2	2; 3					
0.064	3	3, 4			0		
0.125	4	4, 5			0		
0.25	5	5, 6	174	1	0	0.0057	0.9943
0.5	6	6, 7	173	76	0	0.4393	0.5575
1	7	7, 8	97	91	0	0.9381	0.0345
2	8	8, 9	6	5	0	0.8333	0.0057
4	9	9; 10	1	1	0	1	0
8	10	10; 11					
16	11	11; 12					
32	12	12; 13					
64	13	13; 14					
128	14	14; 15					
256	15	≥15			0		

MIC Value (ug/mL)	MIC category	Interval	Isolates at the beginning of the MIC scale (*n* at risk)	Isolates with an MIC in the interval (*n* events)	Censored isolates an the end of the MIC scale (*n* censored)	Susceptible isolates whose growth becomes inhibited by AB (hazard function)	All isolates that are still susceptible (survivor function)
Conventional Farms							
0.016	1	1; 2					
0.03	2	2; 3					
0.064	3	3, 4					
0.125	4	4, 5					
0.25	5	5, 6	290	8	0	0.0276	0.9724
0.5	6	6, 7	282	118	0	0.4184	0.5655
1	7	7, 8	164	153	0	0.9329	0.0379
2	8	8, 9	11	10	0	0.9091	0.0034
4	9	9; 10	0	0	0	0.9091	0.0000
8	10	10; 11	1	1		1.0000	0.0000
16	11	11; 12					
32	12	12; 13					
64	13	13; 14					
128	14	14; 15					
256	15	≥15					

Life table describing the number of *E. coli* isolates in each MIC category for chloramphenicol in a sample of 1,381 isolates from conventional (*n*=883) and antimicrobial-free (*n*=498) production systems

			Number			Proportion of	
MIC Value (ug/mL)	**MIC category**	**Interval**	**Isolates at the beginning of the MIC scale (*n* at risk)**	**Isolates with an MIC in the interval (*n* events)**	**Censored isolates an the end of the MIC scale (*n* censored)**	**Susceptible isolates whose growth becomes inhibited by AB (hazard function)**	**All isolates that are still susceptible (survivor function)**
Antimicrobial Free Farms							
2	1	1,2	498	80	0	0.1606	0.8394
4	2	2,3	418	294	0	0.7033	0.249
8	3	3,4	124	105	0	0.8468	0.0382
16	4	4,5	19	3	0	0.1579	0.0321
32	5	5,6	16	14	0	0.875	0.004
>32	6	≥ 6	2	2	2	0	0.004
Conventional Farms							
2	1	1,2	883	79	0	0.0895	0.9105
4	2	2,3	804	502	0	0.6244	0.342
8	3	3,4	302	197	0	0.6523	0.1189
16	4	4,5	105	33	0	0.3143	0.0815
32	5	5,6	72	40	0	0.5556	0.0362
>32	6	≥ 6	32	32	32	0	0.0362

Life table describing the number of *E. coli* isolates in each MIC category for ampicillin in a sample of 1,381 isolates from conventional (*n*=883) and antimicrobial-free (*n*=498) production systems

			Number			Proportion of	
MIC Value (ug/mL)	**MIC category**	**Interval**	**Isolates at the beginning of the MIC scale (*n* at risk)**	**Isolates with an MIC in the interval (*n* events)**	**Censored isolates an the end of the MIC scale (*n* censored)**	**Susceptible isolates whose growth becomes inhibited by AB (hazard function)**	**All isolates that are still susceptible (survivor function)**
Antimicrobial Free Farms							
1	1	1,2	498	52	0	0.1044	0.8956
2	2	2,3	446	213	0	0.4776	0.4679
4	3	3,4	233	152	0	0.6524	0.1627
8	4	4,5	81	15	0	0.1852	0.1325
16	5	5,6	66	1	0	0.0152	0.1305
32	6	6,7	65	2	0	0.0308	0.1265
>32	7	≥ 7	63	63	63	0	0.1265
Conventional Farms							
1	1	1,2	883	102	0	0.1155	0.8845
2	2	2,3	781	328	0	0.42	0.513
4	3	3,4	453	220	0	0.4857	0.2639
8	4	4,5	233	18	0	0.0773	0.2435
16	5	5,6	215	2	0	0.0093	0.2412
32	6	6,7	213	3	0	0.0141	0.2378
>32	7	≥ 7	210	210	210	0	0.2378

Life table describing the number of *E. coli* isolates in each MIC category for gentamicin in a sample of 1,381 isolates from conventional (*n*=883) and antimicrobial-free (*n*=498) production systems s

			Number			Proportion of	
MIC Value (ug/mL)	**MIC category**	**Interval**	**Isolates at the beginning of the MIC scale (*n* at risk)**	**Isolates with an MIC in the interval (*n* events)**	**Censored isolates at the end of the MIC scale (*n* censored)**	**Susceptible isolates whose growth becomes inhibited by AB (hazard function)**	**All isolates that are still susceptible (survivor function)**
Antimicrobial Free Farms							
<=0.25	1		498	74	0	0.1486	0.8514
0.5	3	3, 4	424	333	0	0.7854	0.1827
1	4	4, 5	91	84	0	0.9231	0.0141
2	5	5, 6	7	5	0	0.7143	0.004
4	6	6, 7	2	0	0	0.5	0.004
8	7	7, 8	2	1	0	0.5	0.002
16	8	8, 9	1	0	0	0	0.002
>16	9	≥ 9	1	1	1	0	0
Conventional Farms							
<=0.25	1		883	125	0	0.1416	0.8584
0.5	3	3, 4	758	598	0	0.7889	0.1812
1	4	4, 5	160	145	0	0.9063	0.017
2	5	5, 6	15	3	0	0.2	0.0136
4	6	6, 7	12	0	0	0.2	0.0079
8	7	7, 8	12	5	0	0.4167	0.0079
16	8	8, 9	7	3	0	0.4286	0.0045
>16	9	≥ 9	4	4	4	0	0.0045

APPENDIX E

Questionnaire:

Antibiotic Usage and Risk Factors for Antimicrobial Resistance in Pork Production

Farm ID Number: _____
Type of farm: 1 □ Antibiotic free, not organic, 2 □ Antibiotic free, organic, 3 □ Not antibiotic free

Date of interview: _____

Herd Information.

How many years has your farm been antibiotic free: _____years.
If your farm is organic, how many years has your farm been organic: _____years.

1 □ Open herd, 2 □ Closed herd.

If open herd, bring in: 1 □ breeding stock, 2 □ nursery pigs, 3 □ feeder pigs
4 □ finishers, 5 □ other: _____

Type of operation: 1 □ farrow to weaning, 2 □ farrow to finish,
3 □ farrow to feeder, 4 □ grower & finishing

Parents.
Genetic line: Sire: _____ Dam: _____
Total number of sows: _____
Total number of boars: _____

Growing pigs.
Total number of pigs marketed per year: _____

Months that pigs are marketed/# marketed per incidence: _____

Total number of growing pigs at any one time: _____

Environment of pigs.

Housing type for breeding animals:
_____
1 □ total confinement, 2 □ partial confinement, 3 □ pasture

Breeding barn: _____
1 □ stalls, 2 □ pens, 3 □ both

Gestation:
_____
1 □ stalls, 2 □ pens, 3 □ both

Farrowing: _____
1 □ crates, 2 □ individual pens, 3 □ group pens, 4 □ hutches

Housing type for growing animals:
Nursery: 1 □ total confinement, 2 □ partial confinement, 3 □ pasture
Floor type:1 □ solid floor, 2 □ slats, 3 □ partial slats
Flooring type: 1 □ concrete, 2 □ metal, 3 □ dirt, 4 □ wood

Grower: 1 □ total confinement, 2 □ partial confinement, 3 □ pasture
Floor type: 1 □ solid floor, 2 □ slats, 3 □ partial slats
Flooring type: 1 □ concrete, 2 □ metal, 3 □ dirt, 4 □ wood

Finisher: 1 □ total confinement, 2 □ partial confinement, 3 □ pasture
Floor type: 1 □ solid floor, 2 □ slats, 3 □ partial slats
Flooring type: 1 □ concrete, 2 □ metal, 3 □ dirt, 4 □ wood

Bedding:
Breeding stock: 1 □ none, 2 □ straw, 3 □ wood shavings, 4 □ saw dust,
5 □ rice hulls 6 □ corn stalks
Nursery: 1 □ none, 2 □ straw, 3 □ wood shavings, 4 □ saw dust,
5 □ rice hulls 6 □ corn stalks
Grower: 1 □ none, 2 □ straw, 3 □ wood shavings, 4 □ saw dust,
5 □ rice hulls 6 □ corn stalks
Finisher: 1 □ none, 2 □ straw, 3 □ wood shavings, 4 □ saw dust,
5 □ rice hulls 6 □ corn stalks

Manure handling:
Finisher:
_____
1 □ pit holding, 2 □ mechanical scraper, 3 □ flush - open gutter, 4 □ flush - under slats, 5 □ hand-cleaned, 6□ shallow pig with scraper

Do you spread manure on fields with livestock:1 □ Yes, 2 □ No, 3 □ other

Ventilation:
Breeding/Gestation:

_____

1 □ natural, 2 □ mechanical, 3 □ both

Nursery:

_____

1 □ natural, 2 □ mechanical, 3 □ both

Grower:

_____

1 □ natural, 2 □ mechanical, 3 □ both

Finisher:

_____

1 □ natural, 2 □ mechanical, 3 □ both

Pig Density:
What is the pig density on your farm: _____

Finisher: _____

Niman Ranch requirements:_____

If pig density not known:

Finisher: 1 □ less 10, 2 □ 11-15, 3 □ 16-20, 4 □ 21-25, 5 □ 26-30,
6 □ 31-35, 7 □ 36-40, 8 □ 41-45, 9 □ 46-50, 10 □ 50+

Finisher: pen length (feet): _____ pen width (feet): _____

Are pigs co-mingled during nursery thru finisher: 1 □ yes, 2□ no, 3□ unsure
If yes, how many times: _____

Feeding of animals:

Finisher: 1 □ feeders, 2 □ floor, 3 □ both

Number of diets:
Finisher:

_____

1 □, 2 □, 3 □, 4 □, 5 □ other

Feed:

_____

1 □ mix on farm, 2 □ by premixed, 3 □, buy corn
4 □ buy soybean 5 □ other

Other animals:

Do you have other livestock besides pigs: 1 □ yes, 2□ no

If yes, what other animals:

_____

Do pigs interact with other livestock: _____

1 □ Yes, 2 □ No, 3 □,Other

Do you have a confinement operation for pigs on site: 1 □ yes,2□ no

Do pigs have fence contact with others : 1 □ Yes, 2 □ No, 3 □,maybe

MEDICATION HISTORY:

Grower/Finishers:

_____

1 □ dewormer, 2 □ mange/lice, 3 □ abx in feed, 4 □ abx in water, 5 □ abx oral, 6 □ abx injection, 7 □ probiotics

List of antibiotics – Grower/finisher pigs:

Antibiotic	Y	N	Primary reason. Use code below	Days in feed	Dose (g/ton)
1- 3 Nitro (Roxarzone)	□	□			
2- Ampicillin	□	□			
3- Apralan (Apramycin)	□	□			
4- ASP (chlortetracycline/Sulfamethazine/Penicillin)	□	□			

5- Aureomycin, CTC (chlortetracycline)	□ □			
6- BMD (Bacitracin)	□ □			
7- CSP (Chlortetracycline/Sulfathiazole/Penicillin)	□ □			
8- Denagard (Tiamulin)	□ □			
9- Erythromycin	□ □			
10- Flavomycin (Bambermycin)	□ □			
11- Gentocin (Gentomycin)	□ □			
12- Hygromix (hygromycin)	□ □			
13- Lincomix, Safeguard (Lincomycin)	□ □			
14- LS 50 (Lincomycin/Spectiniomycin)	□ □			
15- Mecadox (Carbadox)	□ □			
16- Naxcel (Ceftiofur)	□ □			
17- Neomix (neomycin)	□ □			
18- NeoTerra (Neomycin Terramycin)	□ □			
19- OM-5 premix (Oleandomycin)	□ □			
20- Oxytet (oxytetracycline)	□ □			
21- Penicillin and Spectomycin,	□ □			
22- Penicillin G	□ □			
23- Producil (Efromycin)	□ □			
24- Pulmotil (Tilmicosin)	□ □			
25- Stafac (Virginiamycin)	□ □			
26- Sulfachlorpyridazine	□ □			
27- Sulfadimethoxine	□ □			
28- Tetracycline	□ □			
29- Tylan (Tylosin)	□ □			
30- Tylan 40 Sulfa-G (Tylosin/Sulfamethazine)	□ □			
31- Other specify	□ □			
32- Other specify	□ □			
33- Other specify	□ □			
		1 = Growth promotion 2 = Disease prevention 3 = other treatments (specify in column)		

Preventive medicine.
Vaccinations in finishers:

Do you vaccinate finishers against one or more of the following diseases:

Diseases	Vaccinate Y	Vaccinate N	Name of vaccine	Manufacturer of vaccine
Pseudorabies	□	□		
PRRS	□	□		
Swine Flu	□	□		
Salmonella	□	□		
Erysipelas	□	□		
Mycoplasma	□	□		
Atrophic rhinitis	□	□		
E. coli	□	□		
Other_____ _____	□	□		

Pig flow in Finisher:
_____
1 □ continual flow, 2 □ all pigs removed, no cleaning, 3 □ AIAO by room, 4 □ AIAO by building, 5 □ AIAO by site , 6 □ all pigs removed and cleaned, , 7 □ other

Biosecurity.
Visitors to farm:
_____
1 □ take shower, 2 □ clean boots and coveralls, 3 □ 24 hours or longer "pig free"

What type of restroom is available for workers/visitors:
_____
1 □ toilet/septic system 2 □ toilet/municipal sewage system 3 □ outhouse 4 □ no facilities

Are rendering trucks allowed on the farm: 1 □ yes, 2 □ no, 3□ sometimes

Do you control rodents:
_____
1 □ cats, 2 □ dogs, 3 □ traps, 4 □ bait/poison, 5 □ professional exterminator, 6 □ no

Are your buildings bird proof: 1 □ yes, 2 □ no, 3 □ don't know.

Isolation of new breeding stock:

_____

1 □ all, 2 □ some, 3 □ none

Acclimation: 1 □ feedback of feces,
2 □ feedback of mummies, placenta or stillborn piglets, 3 □ exposure to cull animals,
4 □ exposure to sick pigs, 5 □ administration of vaccines.

Disease history.

Grower/finisher pigs:

In the last 12 months, which of the following disease problems were present in one or more grower/finisher pigs.

		Diagnosed by veterinarian.
APP (Actinobacillus pleuropneumonia) (Haemophilus)	□ Yes □ No	□ Yes □ No
PRRS (porcine reproductive and respiratory syndrome)	□ Yes □ No	□ Yes □ No
Swine flu	□ Yes □ No	□ Yes □ No
Salmonella	□ Yes □ No	□ Yes □ No
Glasser's disease (Haemophilus parasuis)	□ Yes □ No	□ Yes □ No
Mycoplasma pneumonia	□ Yes □ No	□ Yes □ No
Circovirus or PMWS (Post-weaning Multisystemic Wasting Syndrome)	□ Yes □ No	□ Yes □ No
Swine dysentery	□ Yes □ No	□ Yes □ No
Atrophic rhinitis	□ Yes □ No	□ Yes □ No
Pseudorabies	□ Yes □ No	□ Yes □ No
Hemorrhagic bowel syndrome	□ Yes □ No	□ Yes □ No
Ileitis (Lawsonia intracellularis)	□ Yes □ No	□ Yes □ No
Gastric ulcers	□ Yes □ No	□ Yes □ No
Erysipelas	□ Yes □ No	□ Yes □ No
Other _____ __	□ Yes □ No	□ Yes □ No

APPENDIX F

Summary of management factors on 35 antimicrobial-free and 60 conventional swine farms in the Midwest U.S.

Variable Name	Description	Category	No. herds	Proportion
A. Biosecurity risk value				
visitoronfarm_0	no visitor biosecurity measures	0	6	6.3
visitoronfarm_1	visitors clean boots	1	61	64.2
visitoronfarm_2	visitors shower and/or visitor is 24 hours free from pig exposure	2	28	29.5
toilet_0	no restroom available for workers/visitors	0	38	40
toilet_1	septic system, municipal sewage or outhouse	1	57	60
exterm	exterminator and/or baits used	0,1	78	82
rendering	allow trucks on farm or not	0,1	21	22.1
birdproof	are buildings bird proof or not	0,1	41	43.2
free_roam	are animals allow to free roam on farm or not	0,1	20	21.1
chickens	are their chickens on farm or not	0,1	17	17.9
newlivestock	are new breeding stock isolated or not	0,1	31	32.6

Variable Name	Description	Category	No. herds	Proportion
animal_contact	do animals have fence contact with others or not	0,1	17	17.9
acclim_0	acclimation is not used	0	43	45.3
acclim_1	acclimation by administration of vaccines	1	17	17.9
acclim_2	use of mummies, cull animals, sick animals or feces	2	35	36.8
B. Disease History for the last 12 months				
actino	Actinobacillus pleuropneumoniae (Haemophilus)	0,1	5	5.3
prrs	Porcine reproductive and respiratory syndrome	0,1	24	25.3
swineflu	Swine Flu (traditional)	0,1	30	31.6
salm	Salmonella	0,1	6	6.3
glassers	Glasser's disease (Haemophilus parasuis)	0,1	11	11.6
myco_pn	Mycoplasma pneumonia	0,1	26	27.4
rhin	Atrophic rhinitis	0,1	5	5.3
hbs	Hemorrhagic bowel syndrome	0,1	28	29.5
ili	Ileitis (Lawsonia intracellularis)	0,1	31	32.6

Variable Name	Description	Category	No. herds	Proportion
ulcer	Gastric Ulcers	0,1	15	15.8
erysip	Erysipelas	0,1	9	9.5
C. Vaccine Usage on farm				
vaccine	any vaccine usage as a preventive treatment or not	0,1	19	20
pseudovx	use of Pseudorabies vaccine or not	0,1	9	9.5
mycovx	use of Mycoplasma or not	0,1	10	10.5
D. Management Practices on the farm				
mixfarm	farms mix own feed on farm or not	0,1	66	69.5
premix	farms buy premixed feed or not	0,1	39	41.1
corn	farms buy corn	0,1	14	14.7
soybean	farms buy soybean product	0,1	31	32.6
manurespread	farms spread manure on fields with livestock or not	0,1	12	12.6
house_1	total confinement is referent (also total and hoop)	1	40	42.1
house_2	partial confinement	2	20	21.1
house_3	Pasture	3	6	6.3
house_4	Hoop barn	4	25	26.3
house_5	Other	5	4	4.2

Variable Name	Description	Category	No. herds	Proportion
flooring	concrete floor is referent or dirt and other	0,1	16	16.8
floor	solid floor is referent or slats, weaved, and other	0,1	50	52.6
bedding_0	no bedding materials used	0	43	45.3
bedding_1	Straw	1	38	40
bedding_2	corn stalks or other	2	14	14.7
vent_0	ventilation to outside	0	33	34.7
vent_1	mechanical or natural and mechanical ventilation	1	31	32.6
vent_2	only natural ventilation	2	31	32.6
aiao	pig flow was continuous or some form of all in and all out (aiao)	0,1	73	76.8
	E. Medication History			
dewormer	use of dewormer or not	0,1	39	41.1
mangelice	use of topical products for mange and lice or not	0,1	14	14.7
probiotics	use of probiotics or not	0,1	13	13.7

APPENDIX G

Appendix G-1. Potential risk factors for azithromycin resistance in *Campylobacter* isolates in finishing pigs from 95 farms in the Midwest, using population averaged logistic regression. Herd type was included in each bivariate analysis.

Variable Name	B (SE)	p-value	Wald p (df)
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	0.38 (0.89)	0.67	
_visitoronfarm_2	0.91 (0.91)	0.32	0.33 (2)
_toilet_1	0.48(0.38)	0.21	
exterm	0.31 (0.45)	0.49	
rendering	-0.38 (0.46)	0.41	
birdproof	0.75 (0.09)	0.09	
free_roam	0.28 (0.41)	0.48	
chickens	-0.38(0.47)	0.42	
newlivestock	-0.41 (0.39)	0.29	
animal_contact	-0.80(0.47)	0.09	
_acclim_0	_	_	
_acclim_1	0.68 (0.57)	0.24	
_acclim_2	0.25 (0.40)	0.54	0.49 (2)
B. Disease History for the last 12 months			
Action	-0.04 (0.90)	0.97	
Prrs	-0.69 (0.46)	0.13	
swineflu	-0.38 (0.41)	0.34	
salm	-0.87 (0.64)	0.17	
glassers	-0.12 (0.43)	0.78	
myco_pn	-0.38 (0.43)	0.38	
rhin	-1.87 (0.55)	0.001	
hbs	0.24(0.40)	0.54	
ili	-0.17 (0.41)	0.67	
ulcer	0.05 (0.54)	0.93	
erysip	-0.40 (0.44)	0.36	
C. Vaccine Usage on farm			
vaccine	0.28 (0.42)	0.51	
pseudovx	-0.14 (0.67)	0.84	
mycovx	-0.09(0.49)	0.86	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
mixfarm	-1.09(0.39)	0.005	
premix	0.24 (0.38)	0.52	
corn	0.73 (0.48)	0.13	
soybean	0.24 (0.38)	0.54	
manurespread	-0.01 (0.49)	0.98	
_house_1	_	_	
_house_2	-0.44(0.52)	0.4	
_house_3	-0.02 (0.58)	0.97	
_house_4	-0.79 (0.51)	0.12	
_house_5	-0.60 (0.70)	0.39	0.52 (4)
flooring	0.12 (0.42)	0.78	
floor	0.52 (0.44)	0.24	
_Ibedding_0	_	_	
_Ibedding_1	-0.28 (0.42)	0.5	
_Ibedding_2	-0.35 (0.59)	0.55	0.75 (2)
_Ivent_0	_	_	
_Ivent_1	-0.32 (0.53)	0.55	
_Ivent_2	0.74 (0.48)	0.13	0.05 (2)
Aiao	0.12 (0.44)	0.78	
E. Medication History			
Dewormer	-0.39 (0.38)	0.3	
Mangelice	-0.52(0.61)	0.39	
Probiotics	-0.91 (0.48)	0.06	

B = Regression coefficient. SE = Standard error.
†A value of $P \leq 0.05$ was considered significant.

Appendix G-2. Potential risk factors for tetracycline resistance in *Campylobacter* isolates in finishing pigs from 95 farms in the Midwest

Variable Name	**B (SE)**	**p-value†**	**Wald p† (df)**
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	-0.66(0.62)	0.29	
_visitoronfarm_2	-1.08 (0.68)	0.11	0.25 (2)
_toilet_0	_	_	
_toilet_1	0.07 (0.36)	0.84	
exterm	-0.68(0.39)	0.08	
rendering	0.02(0.42)	0.96	
birdproof	-0.64(0.51)	0.21	
free_roam	-0.66 (0.34)	0.06	
chickens	-0.53(0.41)	0.20	
newlivestock	0.25 (0.30)	0.42	
animal_contact	-0.06 (0.40)	0.88	
_acclim_0	_	_	
_acclim_1	0.43 (0.41)	0.30	
_acclim_2	-0.05 (0.35)	0.89	0.49 (2)
B. Disease History for the last 12 months			
Action	0.46 (0.68)	0.50	
Prrs	-0.69 (0.46)	0.13	
Swineflu	-0.54 (0.37)	0.14	
Salm	-0.41 (0.63)	0.52	
Glassers	1.41 (0.86)	0.10	
myco_pn	0.02 (0.43)	0.96	
rhin	-0.20 (0.57)	0.73	
hbs	0.04 (0.39)	0.93	
ili	-0.83 (0.35)	0.02	
ulcer	0.11 (0.48)	0.83	
erysip	-0.47 (0.56)	0.40	
C. Vaccine Usage on farm			
Vaccine	-0.77 (0.48)	0.11	
Pseudovx	-0.18 (0.71)	0.80	
Mycovx	-0.81 (0.57)	0.16	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
Mixfarm	-0.36 (0.33)	0.28	
Premix	-0.33 (0.33)	0.32	
Corn	-0.78 (0.41)	0.05	
Soybean	-0.45 (0.33)	0.16	
Manurespread	0.12 (0.42)	0.77	
_house_1	_	_	
_house_2	0.18 (0.53)	0.73	
_house_3	1.11 (0.55)	0.04	
_house_4	0.25 (0.44)	0.57	
_house_5	0.79 (0.60)	0.19	0.19 (4)
_flooring_0	_	_	
Flooring	0.28 (0.46)	0.54	
_floor_0	_	_	
floor	-1.38 (0.45)	0.00	
_Ibedding_0	_	_	
_Ibedding_1	0.68 (0.42)	0.10	
_Ibedding_2	0.54 (0.57)	0.35	0.26 (2)
_Ivent_0	_	_	
_Ivent_1	-1.03 (0.53)	0.05	
_Ivent_2	-0.96 (0.53)	0.07	0.12 (2)
Aiao	-0.75 (0.45)	0.10	
E. Medication History			
Dewormer	-0.00 (0.35)	1.00	
Mangelice	0.73 (0.44)	0.10	
Probiotics	-0.31 (0.42)	0.45	

See appendix G-1 for key.

Appendix G-3. Potential risk factors for tetracycline resistance in *E. coli* isolates from finishing pigs from 95 farms in the Midwest

Variable Name	B (SE)	p-value†	Wald p† (df)
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	0.05 (0.37)	0.88	
_visitoronfarm_2	0.29 (0.50)	0.57	0.81 (2)
_toilet_0	_	_	
_toilet_1	-0.06 (0.28)	0.83	
Exterm	0.02 (0.34)	0.94	
rendering	0.05 (0.28)	0.86	
Birdproof	0.33 (0.34)	0.33	
free_roam	-0.28 (0.30)	0.35	
Chickens	-0.28 (0.33)	0.39	
Newlivestock	-0.31 (0.30)	0.31	
animal_contact	-0.55 (0.31)	0.08	
_acclim_0	_	_	
_acclim_1	0.78 (0.39)	0.05	
_acclim_2	0.03 (0.29)	0.93	0.09
B. Disease History for the last 12 months			
Action	1.09 (0.72)	0.13	
Prrs	0.13 (0.40)	0.74	
Swineflu	0.33 (0.28)	0.24	
Salm	0.67 (0.65)	0.30	
Glassers	0.39 (0.41)	0.35	
myco_pn	0.93(0.34)	0.01	
Rhin	0.15 (0.44)	0.74	

Variable Name	B (SE)	p-value†	Wald p† (df)
Hbs	-0.24 (0.31)	0.45	
Ili	-0.33 (0.33)	0.31	
Ulcer	-0.06 (0.49)	0.90	
Erysip	-0.07 (0.46)	0.89	
C. Vaccine Usage on farm			
Vaccine	-0.03 (0.30)	0.93	
Pseudovx	0.33 (0.37)	0.37	
Mycovx	0.59 (0.53)	0.27	
D. Management Practices on the farm			
Mixfarm	-0.11(0.38)	0.78	
Premix	0.10 (0.36)	0.77	
Corn	-0.45 (0.35)	0.20	
Soybean	-0.07 (0.25)	0.77	
Manurespread	-0.06 (0.37)	0.87	
_house_1	_	_	
_house_2	-0.39 (0.35)	0.27	
_house_3	-1.04 (0.45)	0.02	
_house_4	-0.19 (0.36)	0.60	
_house_5	0.95 (0.90)	0.29	0.06 (4)
Flooring	-0.39 (0.32)	0.22	
floor	0.56 (0.29)	0.06	
_Ibedding_0	_	_	
_Ibedding_1	-0.53 (0.30)	0.08	
_Ibedding_2	-0.29 (0.40)	0.47	0.20 (2)
_Ivent_0	_	_	
_Ivent_1	0.69 (0.34)	0.04	
_Ivent_2	0.79 (0.38)	0.04	0.05 (2)
Aiao	-0.29 (0.29)	0.32	
E. Medication History			
Dewormer	0.12(0.27)	0.67	
Mangelice	0.51 (0.32)	0.11	
Probiotics	-0.19 (0.37)	0.61	

See appendix G-1 for key.

Appendix G-4. Potential risk factors for streptomycin resistance in among *E. coli* isolates from finishing pigs from 95 farms in the Midwest

Variable Name	B (SE)	p-value†	Wald p† (df)
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	-0.02 (0.35)	0.96	
_visitoronfarm_2	0.06 (0.37)	0.87	0.91 (2)
toilet_0	_	_	
toilet_1	0.07 (0.18)	0.70	
exterm	-0.13 (0.25)	0.60	
rendering	-0.07 (0.20)	0.71	
birdproof	-0.26 (0.21)	0.21	
free_roam	-0.34 (0.20)	0.08	
chickens	0.04 (0.27)	0.89	
newlivestock	0.11 (0.19)	0.57	
animal_contact	-0.19 (0.32)	0.55	
_acclim_0	_	_	
_acclim_1	-0.14 (0.26)	0.57	
_acclim_2	-0.10 (0.19)	0.61	0.81 (2)
B. Disease History for the last 12 months			
Action	-0.42 (0.44)	0.33	
Prrs	-0.41 (0.22)	0.06	
Swineflu	-0.51 (0.21)	0.01	
Salm	-0.14 (0.35)	0.69	
Glassers	-0.07(0.30)	0.81	
myco_pn	0.01 (0.20)	0.96	
rhin	-0.44 (0.44)	0.33	
hbs	-0.28 (0.19)	0.14	
ili	-0.26 (0.19)	0.17	
ulcer	-0.71 (0.24)	0.00	
erysip	-0.34 (0.25)	0.17	
C. Vaccine Usage on farm			
Vaccine	-0.12 (0.24)	0.60	
Pseudovx	0.42 (0.29)	0.15	
Mycovx	-0.14 (0.34)	0.68	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
Mixfarm	0.20 (0.19)	0.28	
Premix	-0.23 (0.18)	0.21	
Corn	0.07 (0.18)	0.69	
Soybean	-0.01 (0.18)	0.96	
Manurespread	-0.24 (0.25)	0.33	
_house_1	_	_	
_house_2	0.09 (0.25)	0.70	
_house_3	-0.44 (0.41)	0.28	
_house_4	-0.04 (0.23)	0.87	
_house_5	0.57 (0.18)	0.00	0.00 (4)
Flooring	0.38 (0.23)	0.10	
floor	-0.04 (0.25)	0.86	
_Ibedding_0	_	_	
_Ibedding_1	-0.20 (0.22)	0.38	
_Ibedding_2	-0.09 (0.24)	0.70	0.67 (2)
_Ivent_0	_	_	
_Ivent_1	0.14 (0.25)	0.58	
_Ivent_2	-0.02 (0.25)	0.92	0.69 (2)
Aiao	-0.25 (0.19)	0.18	
E. Medication History			
Dewormer	0.20 (0.19)	0.30	
Mangelice	0.34 (0.20)	0.09	
Probiotics	-0.16 (0.27)	0.56	

See appendix G-1 for key.

Appendix G-5**.** Potential risk factors for ampicillin resistance of among *E. coli* isolates from finishing pigs on 95 farms in the Midwest.

Variable Name	**B (SE)**	**p-value†**	**Wald p† (df)**
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	-0.34(0.47)	0.47	
_visitoronfarm_2	-0.52 (0.49)	0.29	0.50 (2)
toilet_0	_	_	
toilet_1	-0.07 (0.21)	0.74	
Exterm	-0.24 (0.31)	0.44	
rendering	-0.03 (0.24)	0.91	
Birdproof	-0.12 (0.29)	0.67	
free_roam	0.07 (0.26)	0.78	
Chickens	-0.11 (0.33)	0.75	
Newlivestock	0.19 (0.27)	0.47	
animal_contact	0.18 (0.36)	0.62	
_acclim_0	_	_	
_acclim_1	-0.24 (0.30)	0.44	
_acclim_2	0.21 (0.23)	0.36	0.32 (2)
B. Disease History for the last 12 months			
Action	0.58 (0.59)	0.32	
Prrs	-0.10 (0.26)	0.70	
Swineflu	-0.29 (0.25)	0.24	
Salm	0.19 (0.41)	0.65	
Glassers	0.08 (0.40)	0.84	
myco_pn	0.37 (0.26)	0.16	
Rhin	-1.71 (0.44)	0.00	
Hbs	0.36 (0.24)	0.13	
Ili	0.18 (0.22)	0.41	
Ulcer	-0.08(0.33)	0.82	
Erysip	-0.36 (0.27)	0.18	
C. Vaccine Usage on farm			
Vaccine	0.06 (0.21)	0.78	
Pseudovx	0.21 (0.28)	0.45	
Mycovx	-0.06 (0.29)	0.84	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
Mixfarm	0.39 (0.24)	0.10	
Premix	-0.30 (0.22)	0.17	
Corn	-0.18(0.29)	0.52	
Soybean	0.05 (0.24)	0.83	
Manurespread	-0.41 (0.30)	0.17	
_house_1	_	_	
_house_2	0.04 (0.31)	0.89	
_house_3	-0.57 (0.52)	0.28	
_house_4	0.27 (0.29)	0.36	
_house_5	-0.23 (0.85)	0.78	0.51 (4)
Flooring	0.43 (0.27)	0.11	
floor	-0.34 (0.30)	0.25	
_Ibedding_0	_	_	
_Ibedding_1	0.07 (0.32)	0.83	

Table 4.7a (continued)

Variable Name	B (SE)	p-value	Wald p (df)
_Ibedding_2	0.43 (0.34)	0.21	0.43 (2)
_Ivent_0	_	_	
_Ivent_1	0.45 (0.42)	0.29	
_Ivent_2	0.10 (0.42)	0.82	0.27 (2)
Aiao	-0.14 (0.26)	0.60	
E. Medication History			
Dewormer	0.14 (0.25)	0.57	
Mangelice	0.18 (0.31)	0.57	
Probiotics	0.45 (0.37)	0.23	

See appendix G-1 for key.

Appendix G-6. Potential risk factors for ampicillin resistance of sulfamethoxazole in *E. coli* isolates from finishing pigs on 95 farms in the Midwest

Variable Name	**B (SE)**	**p-value†**	**Wald p† (df)**
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	-0.30 (0.29)	0.30	
_visitoronfarm_2	-0.25 (0.32)	0.45	0.58 (2)
toilet_0	_	_	
toilet_1	-0.27 (0.18)	0.14	
exterm	-0.12 (0.23)	0.60	
rendering	0.04 (0.20)	0.86	
birdproof	0.09 (0.21)	0.67	
free_roam	-0.13 (0.22)	0.56	
chickens	0.11 (0.26)	0.69	
newlivestock	-0.15 (0.20)	0.45	
animal_contact	-0.18 (0.24)	0.44	
_acclim_0	_	_	
_acclim_1	-0.10 (0.22)	0.64	
_acclim_2	-0.06 (0.20)	0.77	0.89 (2)
B. Disease History for the last 12 months			
Actino	-0.13 (0.37)	0.72	
Prrs	-0.05(0.22)	0.84	
Swineflu	-0.13 (0.20)	0.51	
Salm	0.10 (0.29)	0.72	
Glassers	-0.11 (0.29)	0.70	
myco_pn	0.17 (0.20)	0.38	
rhin	-0.09 (0.26)	0.73	
hbs	-0.16 (0.21)	0.43	
ili	0.10 (0.20)	0.61	
ulcer	-0.46 (0.25)	0.07	
erysip	-0.25 (0.26)	0.34	
C. Vaccine Usage on farm			
Vaccine	0.11 (0.22)	0.63	
Pseudovx	0.53 (0.29)	0.06	
Mycovx	0.25 (0.28)	0.37	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
Mixfarm	0.24 (0.21)	0.24	
Premix	0.07 (0.19)	0.73	
Corn	-0.15 (0.25)	0.54	
Soybean	0.10 (0.19)	0.61	
Manurespread	-0.28 (0.22)	0.20	
_house_1	_	_	
_house_2	0.04 (0.24)	0.86	
_house_3	-0.73 (0.41)	0.07	
_house_4	-0.29 (0.23)	0.20	
_house_5	0.52 (0.20)	0.01	0.00 (4)
Flooring	-0.10 (0.27)	0.70	
floor	0.09 (0.22)	0.70	
_Ibedding_0	-	_	
_Ibedding_1	-0.11(0.21)	0.61	
_Ibedding_2	-0.38 (0.24)	0.11	0.28 (2)
_Ivent_0	_	_	
_Ivent_1	0.33 (0.26)	0.22	
_Ivent_2	0.27 (0.27)	0.31	0.45 (2)
aiao	-0.16 (0.19)	0.41	
E. Medication History			
dewormer	0.32 (0.20)	0.10	
mangelice	0.55 (0.23)	0.02	
probiotics	0.01 (0.31)	0.98	

See appendix G-1 for key.

Appendix G-7**.** Potential risk factor for chloramphenicol resistance of in *E. coli* isolates from finishing pigs on 95 farms in the Midwest

Variable Name	**B (SE)**	**p-value†**	**Wald p† (df)**
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	-0.60 (0.56)	0.29	
_visitoronfarm_2	-0.42 (0.59)	0.47	0.54 (2)
toilet_0	_	_	
toilet_1	-0.04 (0.33)	0.90	
exterm	0.62 (0.47)	0.19	
rendering	-1.50 (0.52)	0.00	
birdproof	0.40 (0.42)	0.34	
free_roam	0.16 (0.33)	0.64	
chickens	1.17 (0.33)	0.00	
newlivestock	-0.07 (0.36)	0.84	
animal_contact	-0.09 (0.60)	0.88	
_acclim_0	_	_	
_acclim_1	-0.28 (0.44)	0.53	
_acclim_2	0.04 (0.34)	0.91	0.78 (2)
B. Disease History for the last 12 months			
actino	0.00 (0.41)	0.99	
Prrs	0.18 (0.36)	0.62	
Swineflu	-0.04 (0.33)	0.91	
Salm	-0.78 (0.74)	0.29	
Glassers	0.33 (0.45)	0.46	
myco_pn	0.11 (0.36)	0.75	
Rhin	-0.71 (0.74)	0.34	
Hbs	0.23 (0.35)	0.51	
Ili	0.33 (0.32)	0.30	
Ulcer	-0.31 (0.43)	0.48	
Erysip	-1.09 (0.57)	0.06	
C. Vaccine Usage on farm			
Vaccine	0.08 (0.37)	0.82	
Pseudovx	0.81 (0.40)	0.05	
Mycovx	0.51 (0.40)	0.20	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
Mixfarm	0.12 (0.33)	0.70	
Premix	0.37 (0.33)	0.25	
Corn	-0.16 (0.41)	0.70	
Soybean	0.00 (0.32)	1.0	
Manurespread	-0.44 (0.45)	0.34	
_house_1	_	_	
_house_2	0.07 (0.47)	0.88	
_house_3	-0.10 (0.57)	0.86	
_house_4	-0.86 (0.48)	0.08	
_house_5	-0.39 (0.80)	0.71	0.40 (4)
Flooring	-0.47 (0.44)	0.28	
Floor	0.27 (0.44)	0.54	
_Ibedding_0	_	_	
_Ibedding_1	0.05 (0.42)	0.90	
_Ibedding_2	-1.90 (0.71)	0.01	0.02 (2)
_Ivent_0	_	_	
_Ivent_1	1.25 (0.51)	0.02	
_Ivent_2	1.61 (0.47)	0.00	0.00(2)
Aiao	-0.04 (0.42)	0.93	
E. Medication History			
Dewormer	0.46 (0.34)	0.17	
Mangelice	0.32 (0.34)	0.34	
Probiotics	-0.35 (0.51)	0.49	

See appendix G-1 for key.

Appendix G-8. Potential risk factors multidrug resistance in *Campylobacter* isolates from finishing pigs on 95 farms in the Midwest. Herd type was included in each analysis.

Variable Name	**B (SE)**	**p-value†**	**Wald p† (df)**
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	-0.32 (0.75)	0.66	
_visitoronfarm_2	0.22 (0.78)	0.77	0.35 (2)
_toilet_1	0.12 (0.35)	0.74	
Exterm	0.13 (0.43)	0.77	
rendering	-0.59 (0.44)	0.18	
Birdproof	0.58 (0.43)	0.17	
free_roam	0.02 (0.40)	0.96	
Chickens	-0.32 (0.44)	0.46	
Newlivestock	-0.53 (0.37)	0.16	
animal_contact	-0.60 (0.43)	0.17	
_acclim_0	_	_	
_acclim_1	0.55 (0.52)	0.29	
_acclim_2	0.10 (0.38)	0.80	0.57 (2)
B. Disease History for the last 12 months			
Action	0.21 (0.95)	0.83	
Prrs	-0.56 (0.44)	0.21	
Swineflu	-0.21 (0.37)	0.58	
Salm	-0.66 (0.58)	0.26	
Glassers	-0.02 (0.42)	0.95	
myco_pn	-0.41 (0.41)	0.31	
rhin	-1.95 (0.55)	0.000	
hbs	0.31 (0.38)	0.73	
ili	0.03 (0.38)	0.94	
ulcer	-0.04 (0.53)	0.93	
erysip	-0.62 (0.57)	0.28	
C. Vaccine Usage on farm			
Vaccine	0.12 (0.46)	0.79	
Pseudovx	-0.21 (0.66)	0.75	
Mycovx	-0.15 (0.48)	0.76	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
Mixfarm	-0.78 (0.36)	0.03	
Premix	0.21 (0.36)	0.56	
Corn	0.60 (0.47)	0.20	
Soybean	0.22 (0.36)	0.55	
Manurespread	-0.09 (0.47)	0.84	
_house_1	_	_	
_house_2	-0.08 (0.42)	0.84	
_house_3	-0.30 (0.63)	0.64	
_house_4	-0.56 (0.49)	0.25	
_house_5	-0.34 (0.69)	0.62	0.68 (4)
Flooring	0.35 (0.43)	0.41	
floor	0.43 (0.39)	0.27	
_Ibedding_0	_	_	
_Ibedding_1	-0.37 (0.38)	0.33	
_Ibedding_2	0.02 (0.62)	0.97	0.51 (2)
_Ivent_0	_	_	
_Ivent_1	-0.38 (0.52)	0.45	
_Ivent_2	0.39 (0.47)	0.42	0.19 (2)
Aiao	0.26 (0.40)	0.51	
E. Medication History			
Dewormer	-0.26 (0.38)	0.47	
Mangelice	-0.36 (0.46)	0.43	

See appendix G-1 for key.

Appendix G-9**.** Potential risk factor for multidrug resistance in *E. coli* isolates from finishing pigs on 95 farms in the Midwest. Herd type was included in each analysis.

Variable Name	**B (SE)**	**p-value†**	**Wald p† (df)**
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	-0.50 (0.33)	0.13	
_visitoronfarm_2	-0.16 (0.36)	0.66	0.10 (2)
_toilet_1	-0.11 (0.18)	0.52	
Exterm	-0.04 (0.23)	0.88	
rendering	-0.01 (0.19)	0.95	
Birdproof	0.13 (0.25)	0.59	
free_roam	-0.28 (0.21)	0.19	
Chickens	-0.02 (0.30)	0.94	
Newlivestock	-0.15 (0.22)	0.48	
animal_contact	-0.30 (0.28)	0.30	
_acclim_0	_	_	
_acclim_1	0.07 (0.25)	0.78	
_acclim_2	0.17 (0.20)	0.40	0.70 (2)
B. Disease History for the last 12 months			
Action	0.23 (0.37)	0.54	
Prrs	-0.22 (0.24)	0.37	
Swineflu	-0.48 (0.21)	0.02	
Salm	-0.33 (0.26)	0.21	
Glassers	-0.24 (0.36)	0.51	
myco_pn	0.33 (0.22)	0.14	
Rhin	-0.45 (0.27)	0.10	
Hbs	-0.01 (0.22)	0.96	
Ili	0.10 (0.20)	0.62	
Ulcer	-0.47 (0.28)	0.09	
Erysip	-0.23 (0.30)	0.43	
C. Vaccine Usage on farm			
Vaccine	0.19 (0.24)	0.44	
Pseudovx	0.70 (0.35)	0.05	
Mycovx	0.36 (0.33)	0.27	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
Mixfarm	-0.27 (0.22)	0.23	
Premix	-0.33 (0.20)	0.10	
Corn	0.04 (0.26)	0.89	
Soybean	0.06 (0.19)	0.76	
Manurespread	-0.36 (0.19)	0.06	
_house_1	_	_	
_house_2	-0.15 (0.26)	0.56	
_house_3	-0.79 (0.31)	0.01	
_house_4	-0.18 (0.24)	0.46	
_house_5	-0.13 (0.19)	0.49	0.01 (4)
Flooring	0.08 (0.23)	0.71	
floor	-0.01 (0.33)	0.98	
_Ibedding_0	_	_	
_Ibedding_1	-0.24 (0.25)	0.34	
_Ibedding_2	-0.17 (0.22)	0.45	0.61 (2)
_Ivent_0	_	_	
_Ivent_1	0.60 (0.23)	0.01	
_Ivent_2	0.45 (0.23)	0.05	0.03 (2)
Aiao	-0.21 (0.20)	0.29	
E. Medication History			
Dewormer	0.12 (0.22)	0.57	
Mangelice	-0.26 (0.21)	0.20	

See appendix G-1 for key.

Appendix G-10**.** Potential risk factors for multi-bacterial-antimicrobial resistance in feces samples from finishing pigs on 95 swine farms in the Midwest.

Variable Name	**B (SE)**	**p-value†**	**Wald p† (df)**
A. Biosecurity risk value			
_visitoronfarm_0	_	_	
_visitoronfarm_1	-0.80 (0.51)	0.11	
_visitoronfarm_2	-0.53 (0.58)	0.36	0.24 (2)
toilet_0		_	
toilet_1	-0.25 (0.31)	0.42	
Exterm	-0.11 (0.35)	0.76	
rendering	-0.28 (0.40)	0.48	
Birdproof	0.66 (0.41)	0.11	
free_roam	-0.08 (0.33)	0.82	
Chickens	-0.33 (0.34)	0.33	
Newlivestock	-0.65 (0.30)	0.03	
animal_contact	-0.90 (0.36)	0.01	
_acclim_0	_	_	_
_acclim_1	0.04 (0.43)	0.93	
_acclim_2	-0.22 (0.32)	0.49	0.73 (2)
B. Disease History for the last 12 months			
Action	0.60 (1.11)	0.59	
Prrs	-0.40 (0.45)	0.37	
Swineflu	-0.70 (0.34)	0.04	
Salm	-0.43 (0.65)	0.50	
Glassers	0.93 (0.63)	0.14	
myco_pn	0.32 (0.41)	0.43	
Rhin	-1.30 (0.68)	0.06	
Hbs	0.13 (0.38)	0.73	
Ili	-0.37 (0.36)	0.30	
Ulcer	-0.36 (0.54)	0.50	
Erysip	-0.50 (0.56)	0.37	
C. Vaccine Usage on farm			
Vaccine	-.07 (0.45)	0.88	
Pseudovx	0.57 (0.48)	0.23	
Mycovx	0.08 (0.48)	0.86	

Variable Name	B (SE)	p-value†	Wald p† (df)
D. Management Practices on the farm			
Mixfarm	-0.52 (0.37)	0.16	
Premix	-0.01 (0.34)	0.97	
Corn	0.46 (0.47)	0.33	
Soybean	-0.14 (0.31)	0.65	
Manurespread	-0.11 (0.41)	0.79	
_house_1	_	_	_
_house_2	-0.55 (0.37)	0.13	
_house_3	-0.09 (0.42)	0.84	
_house_4	-0.88 (0.46)	0.05	
_house_5	-0.17 (0.62)	0.78	0.22 (4)
Flooring	0.25 (0.33)	0.45	
floor	0.38 (0.43)	0.37	
_Ibedding_0	_	_	
_Ibedding_1	-0.69 (0.38)	0.07	
_Ibedding_2	-0.55 (0.45)	0.23	0.19 (2)
_Ivent_0	_	_	
_Ivent_1	0.14 (0.41)	0.74	
_Ivent_2	0.64 (0.40)	0.11	0.25 (2)
Aiao	-0.41 (0.31)	0.19	
E. Medication History			
Dewormer	-0.38 (0.30)	0.22	
Mangelice	-0.15 (0.40)	0.70	
Probiotics	-0.67 (0.40)	0.09	

See appendix G-1 for key.

VITA

Name:	Susan Noble Rollo
Address:	Zoonosis Control, Health Service Region 6/5 South Department of State Health Services 5425 Polk St, Suite J Houston, TX 77023
Email Address:	srollodvm@yahoo.com
Education:	B.S. Zoology, Texas Tech University, 1986 M.S., Biology, Texas Tech University, 1989 D.V.M., Texas A&M University, 1993 Ph.D., Texas A&M University 2011