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THE EPIDEMIOLOGY AND MECHANISMS OF REDUCED ANTIMICROBIAL SUSCEPTIBILITY OF *CAMPYLOBACTER SPP.* FROM NORTHEASTERN AND MIDWESTERN DAIRY FARMS IN THE UNITED STATES

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Lisa W. Halbert, DVM

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THE EPIDEMIOLOGY AND MECHANISMS OF REDUCED ANTIMICROBIAL SUSCEPTIBILITY OF *CAMPYLOBACTER SPP.* FROM NORTHEASTERN AND MIDWESTERN DAIRY FARMS IN THE UNITED STATES

By

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ABSTRACT

THE EPIDEMIOLOGY AND MECHANISMS OF REDUCED ANTIMICROBIAL SUSCEPTIBILITY OF *CAMPYLOBACTER SPP.* FROM NORTHEASTERN AND MIDWESTERN DAIRY FARMS IN THE UNITED STATES

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Campylobacter spp are the most common cause of bacterial gastroenteritis in many countries around the world. Outbreaks of Camplobacterosis have been most notably attributed to the consumption of contaminated poultry, raw milk, educational visits to farms, and or can be waterborne. Recently there has been much concern about the documented occurrence of antimicrobial resistance in human *Camploybacter* cases. Since many human cases are acquired via the foodborne or waterborne route, it is prudent to examine food animal production systems which may contribute to the selection of resistance genes in this organism which may either contaminate food products or water through the application of animal manure. *Campylobacter* from dairy sources is very infrequently assessed as to its antimicrobial susceptibility profile despite human cases being attributed to raw milk, educational farm visits, and the potential for dairy cattle manure to contaminate water or other environmental sources.

Therefore, this study was developed with the overall goal of identifying risk factors hat may be explored as possible points of intervention to lessen antimicrobial resistance in *Campylobacter* in dairy cattle. This overall goal was

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addressed through the four following objectives: 1) Compare the patterns of antimicrobial resistance between organic and conventional dairy farm management types 2) Determine individual animal risk factors for decreased susceptibility 3) Determine herd risk factors for antimicrobial decreased susceptibility 4) Determine the mechanism of resistance for tetracycline.

The findings of the following material can be briefly summarized by addressing each objective above. Overall *Campylobacter* from both farm types was susceptible to most antimicrobials. Some resistance was demonstrated to ampicillin, kanamycin, tetracycline, sulfamethoxazole. The proportion of resistant isolates was only significantly higher for *Campylobacter* from conventional farms for tetracycline. Individual animal risk factors primarily include animal type. Calves were significantly at greater odds for decreased susceptibility for kanamycin, tetracycline and ampicillin. Some animal treatments were associated with increased odds of decreased susceptibility. Farm management risk factors that were associated with decreased risk include many of common sense hygiene, such as moving calf hutches in between calves, disinfecting milk buckets, and separating maternity areas from sick cows. The use of some antimicrobials was associated with decreased susceptibility. However, many of the patterns were not clear-cut and may include exposure to drugs other than the antimicrobial of interest in the outcome. It was confirmed that tetracycline resistance was conferred by the genetic determinant *Tet O*. Also several isolates became susceptible during the regrowth period, which supports plasmid carriage.

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INTRODUCTION

RATIONALE

While foodborne bacteria have been causing illness for a millennia, only recently have bacteria which cause gastroenteritis been addressed as an emerging concern. Surveillance systems around the world in many countries now capture data in order to summarize which bacteria a associated with illness and as well as some risk factors such as food sources involved and trends such as changes in antimicrobial susceptibility. Campylobacter is one of the commonest causes of bacterial gastroenteritis globally and is included in many such surveillance systems such as FoodNet in the United States and DANMAP in Denmark.

Although most cases of campylobacterosis are self-limiting and go unreported, the severe cases are serious protracted bouts of bloody diarrhea that may require hospitalization and occasionally death. In the elderly, immunocompromised, or neonatal patients antimicrobial therapy is often warranted. However if the etiologic agent is refractory to the antimicrobials prescribed the duration of illness and secondary cost of such case rise dramatically.

Campylobacterosis is a global problem. In developing nations it claims the lives of many infants which are exposed at an early agent due to the endemic status of the organism in areas where hygiene and medical attention are lacking.

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However, the bacteria does not frequently cause illness in adults due to acquired immunity.

The disease distribution in developed countries is much different. While there are cases in very young children, there is another peak in early adults as they learn how to prepare their own food. Also in developing countries, including the United States and Finland, the majority of antibiotic resistant infections are acquired during travel abroad.

While antimicrobial resistance in Campylobacter and other foodborne bacteria has been identified as an emerging concern, little information on the risk factors contributing to the selection of resistant organisms has been undertaken. Close examination of the data in the United States has demonstrated that key pieces in the selection and dissemination of resistance determinants is missing. While the consumption of chicken has increased in the United States, and chickens can be "flock-medicated" for disease, the incidence rate of human campylobacter cases has declined by more than 26% since surveillance began. Unfortunately too few years have been studied to determine if any differences are occurring in antimicrobial resistance in humans in the United States. However, there have been some trends in antimicrobial resistance increasing in both human campylobacter and animal isolates since the introduction of new drugs such as the fluoroquinolones. Unfortunately, data is lacking on where this resistance is really being selected (i.e. by the selective pressure of very clean, sanitized homes and misuse of antimicrobials by human physicians or through the foodchain by medication of animals).

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Regardless of the voids in available information, one fact remains. Dairy exposure through the consumption of raw milk, petting of animals during educational visits, and the waterborne cases contribute to the majority of the outbreaks of campylobacter. However, very little follow up on the resistance patterns has occurred after these outbreaks to determine the burden caused by Campylobacter acquired by these routes.

Also with the increased consumer interest in minimally processed foods, there have been consumer groups intentionally by-passing safety measures such as pasteurization. Very recently there have been outbreaks in the United States by foodborne bacteria including Salmonella & Campylobacter where people have intentionally consumed raw milk. In some states raw milk may be legally purchased through dairies that are certified to sell the raw product. However, certification does not insure that every glass of milk is pathogen free. In states where raw milk sale is illegal, consumers have by-passed the system to the extent of leasing cows so that the raw milk they consume is "theirs". Such a cow-leasing program was linked to an outbreak of Campylobacterosis in Wisconsin in December 2001.

PROBLEM STATEMENT

From the above it is clear that Campylobacter from dairy sources is still an issue of public health. However the burden of resistance which may occur such as in the cases above has not been ascertained. It may be that due to the limited antimicrobials allowed to dairy farmers and veterinarians, that the selective

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pressure within these animals is low compared to poultry which may be medicated a 10,000 bird flock at a time.

Therefore the dairy industry with its uniquely different management styles allows the opportunity to investigate the epidemiological links between decreased susceptibility and potential herd management and individual animal risk factors.

BASIC RESEARCH QUESTIONS TO BE STUDIED

In this dissertation work the overall aim is to identify risk factors which may be explored as possible points of intervention to help mitigate the antimicrobial resisance in Campylobacter in dairy cattle. In order to address this aim, there are several underlying key research questions that should be answered by the studies conducted. They are:

1) Do organic farms appear to exert less selective pressure on Campylobacter compared to conventional dairy management?
2) Does herd management contribute to decreased antimicrobial susceptibility in dairy cattle?
3) Do patterns of susceptibility differ by animal related factors?
4) How do genetic mechanisms contribute to the observed resistance of Campylobacter isolates?

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HYPOTHESES TO BE TESTED

In order to address the above aim and answer the key research questions, a number of individual hypotheses were developed to be tested. They are:

1) Patterns of antimicrobial susceptibility in Campylobacter spp from organic farms do not differ from isolates from conventional dairy herds.
2) Specific dairy herd management practices are not associated with antimicrobial susceptibility of Campylobacter.
3) Specific individual animal risk factors are not associated with the antimicrobial susceptibility of Campylobacter
4) Antimicrobial susceptibility of Campylobacter to tetracycline does not differ either by exposure to the drug use on the farm or by the genetic carriage of molecular determinants.

OVERVIEW OF RESEARCH

A literature review of the role of dairy sources in human campylobacter infection and antimicrobial resistance of campylobacter in dairy isolates is presented in chapter one. Chapter two addresses hypothesis 1 by describing the patterns of resistance across two main management styles in dairy farming, organic and conventional dairying. Chapter three addresses hypothesis 2 by investigated herd management practices which may be associated with decreased susceptibility in Campylobacter from dairy isolates. Similarly, chapter four addresses hypothesis 3 by evaluate potential risk factors for decreased

susceptibility of campylobacter at the individual animal level. Chapter five is designed to address hypotheses 4 and 5, by using molecular genetic techniques to describe the genetic means of tetracycline resistance in Campylobacter.

CHAPTER ONE

The role of cattle in *Campylobacter spp.* infection in humans and antimicrobial resistance: a review

Abstract

Campylobacter spp are one of the most frequently identified causes of human gastroenteritis worldwide. Since this organism can colonize many warm blooded animals such as cattle without causing infection, food animals are often considered a source of human infection. For these reasons a review of the literature was performed to evaluate three objectives. The first objective was to summarize risk factors for human cases of campylobacterosis. Case-control evaluation of risk factors for infection was most frequently used; however, inconsistencies were evident in the risk factors identified across researchers. The second objective involved case discussion of outbreaks of human Campylobacter directly linked to dairy cattle, either through contact or consumption of dairy products. Consumption of raw milk or contact with farms animals are frequently the point of exposure, although the role of dairy animals in contamination of surface water warrants further study. The third objective was to describe patterns of antimicrobial resistance in Campylobacter isolated from cattle as well as the differing. It was found that much disparity in laboratory

techniques and antimicrobials studied by each research teams makes direct comparisons not feasible.

Introduction

Diarrheal diseases infect more than 1.5 billion people worldwide and claim the lives of approximately 2 million children annually (Acar and Rostel 2001). Worldwide, *Campylobacter* cases outnumber *Salmonella, Shigella* and *E. coli* (Allos 2001). *Campylobacter* is often considered hyperendemic in developing countries due to poor sanitation and presents with both a high incidence of clinical disease, particularly in children, and asymptomatic infections in both children and adults (Hart and Kariuki 1998) (Padungtod and Kaneene 2003). Subsequently, *Campylobacter* is frequently a cause of traveler's diarrhea among visitors to these regions and contributes to a majority of the drug resistant strains that were acquired abroad in residents of developed countries (Hart and Kariuki 1998) (Rautelin, Vierikko et al. 2003).

In the United States alone foodborne disease is estimated to cause 76 million illnesses, of which 325,000 persons require hospitalized, and 5200 die annually. Of these cases of foodborne illness, 2.5 million cases are estimated to be caused by *Campylobacter* (Mead, Slutsker et al. 1999). Since *Campylobacter* infectious are usually mild and self-limiting, the awareness of this organism has taken a back seat to bacteria such as *E coli* 0157:h7 and *Listeria* which can have

much higher case fatality rates, of 8/1000 cases and 200/1000 cases, respectively (Mead, Slutsker et al. 1999). *Campylobacter* can be associated with death in 1/1000 cases. However, *Campylobacter* is associated with 2-5 times as many cases of gastroenteritis as either *Salmonella* or *E. coli* (Mead, Slutsker et al. 1999)

However, other serious disease sequelea can follow gastrointestinal infections with *Campylobacter.* Reactive arthritis or the acute neuropathy, Guillain –Barre' syndrome (GBS) can both be associated to recent infection with *Campylobacter* (Nachamkin, Allos et al. 1998). Estimates of GBS incidence indicate that this syndrome can be manifested 1 person of every 1000 cases of Campylobacter gastroenteritis. This acute neuropathy is caused by an autoimmune response that results in demyelination of both motor and sensory nerves which results in weakness, ataxia and sensory disturbances. Impairment can be so severe that assisted breathing is required and life-long disability may result (Rees, Soudain et al. 1995). For these severe forms of Campylobacterosis in humans, it is critical that appropriate antimicrobial therapy is effective. (Skirrow and Blaser 2000)

Due to the global importance of this foodborne pathogen, it is pertinent to summarize what is known about the role of cattle as a food animal which might play a role in disseminating not only *Campylobacter* infection, but also antimicrobial resistance in these bacteria. The objectives of this literature review

are to 1) to summarize human risk factors for campylobacter infection with the specific aim of identifying the role of cattle in comparison to other routes through which infection with *Campylobacter* have occurred 2) to describe several outbreaks of campylobacterosis which were traced to cattle, their food products, or farm contact and 3) to evaluate the role of cattle in the development of antimicrobial resistance in this foodborne pathogen. The insight provided by such a review should elucidate areas requiring more research so that the role of cattle in human infection and antimicrobial resistance in *Campylobacter* may be mitigated.

Materials and Methods

An initial search of the literature included the utilization of electronic databases including Medline, ISC web, Michigan State University electronic resources including Agricola, Zoological Record using keywords searches of *Campylobacter, C. jejuni,* campylobacterosis, beef, raw milk, cattle, antimicrobial resistance, and antibiotic resistance. Emphasis was placed on peer-review publications. References cited by authors which were pertinent to the above topics were also obtained and reviewed. However, due to the sparseness of material for antimicrobial resistance in Campylobacter isolated from cattle, reports from surveillance data and abstracts from international conferences was also utilized.

Risk factors for human campylobacterosis

Risk factors for *Campylobacter* infection have been identified by various researchers around the world, primarily using case-control studies. In Norway, sporadic cases of gastroenteritis due to *Campylobacter* was associated with drinking undisinfected water, living on a farm (including daily contact with ruminant farm animals), drinking unpasteurized milk, eating at barbeques, eating poultry that was purchased raw, having occupational exposure to animals, and eating undercooked pork (Kapperud, Espeland et al. 2003). Interestingly, the consumption of poultry products alone was not a significant risk factor nor was consumption of red meats (Kapperud, Espeland et al. 2003). Therefore, the association of gastroenteritis with poultry purchased raw may be due to cross contamination during preparation rather than from direct consumption of poultry. In contrast to findings in the United States, eating outside of the home was not associated with *Campylobacter* infection in Norway (Kapperud, Espeland et al. 2003)

In New Zealand and several European countries seasonal distribution to *Campylobacter* cases has been observed. In Wales and Scotland the seasonal peak in cases was within the latter part of May, whereas cases in Norway peaked in July (Nylen, Dunstan et al. 2002). The most prominent seasonal peak, defined by the proportion of cases occurring within +/- 3 weeks, occurred in Finland and

is believed to be due to cases acquired abroad while on holiday (Nylen, Dunstan et al. 2002). Foreign travel is indeed associated with more resistant strains of *Campylobacter* than the strains acquired domestically by Finnish residents. (Rautelin, Vierikko et al. 2003). Other potential explanations for the patterns observed in different countries included seasonal prevalence of *Campylobacter* in potential reservoirs and variations in human behavior (Nylen, Dunstan et al. 2002). However, in Norway and Denmark, human cases of campylobacterosis preceded peak prevalence in poultry flocks (Nylen, Dunstan et al. 2002). Similarly, in the United Kingdom, little seasonality of *Campylobacter* carriage in poultry has been observed, despite seasonality of human cases. From the above disparities in epidemiologic trends between humans and the poultry populations in the respective countries, the authors concluded that other ecological niches in the exposure of humans to campylobacter such as wild bird populations, water, and ruminant animals should be explored (Nylen, Dunstan et al. 2002)

In the United Kingdom, case-control analysis of risk factors for *Campylobacter* infection found some constancy with the above studies, but also exposed other discrepancies. Occupational exposure to raw meat, having a household pet with diarrhea, and ingesting surface water were associated with increased risk of infection (Adak, Cowden et al. 1995). However, handling raw chicken in the home, consuming chicken dishes prepared in the home,

occupational exposure to livestock or livestock manure were associated with decreased risk (Adak, Cowden et al. 1995). Also, while consumption of raw milk was associated with higher odds of infection, the association was not statistically significant (p=0.11). (Adak, Cowden et al. 1995) More recent trends in England and Wales from 1995 to 1999 in *Campylobacter* outbreaks have found commercial eateries the most consistent venue accounting for 64% of outbreaks. Animal contact and person to person exposure only were attributed to 1 outbreak each (Frost, Gillespie et al. 2002). From this study, poultry was the food most often implicated (Frost, Gillespie et al. 2002).

When more detailed description of food exposures was determined in a case-control study of human *Campylobacter* infection in New Zealand, some risk factors were identified. (Eberhart-Phillips, Walker et al. 1997) Significantly increased odds were found with consumption of raw or undercooked poultry, chicken eaten at restaurants, overseas travel, rainwater used for home consumption, consumption of raw dairy products, and contact with puppies and calves. However, the consumption of baked or roasted chicken was found to be associated with decreased odds of infection. (Eberhart-Phillips, Walker et al. 1997)

In the United States, risk factors for *Campylobacter* infections have been investigated by various authors. During investigations of 23 outbreaks of campylobacterosis, the consumption of raw milk was attributed to 14 of the

outbreaks. (Finch and Blake 1985). Four outbreaks were attributed to food handler error, such as cross-contamination; whereas, poultry, eggs or beef only accounted for the source of infection in an additional five outbreaks combined. (Finch and Blake 1985) A recent outbreak of *Campylobacter* occurred during a luncheon in which the implicated foods gravy and pineapple led to the investigation of food-handler error (Olsen, Hansen et al. 2001). Pulse-field gel electrophoresis identified that an ill food handler did indeed share the same genetic type as the patients who became ill after attending the luncheon (Olsen, Hansen et al. 2001).

In 2001, Friedman and colleagues found the strongest risk factor for human infection with *Campylobacter* to be foreign travel (Friedman, Neimann et al. 2000). Once foreign travelers were excluded from further analysis, eating undercooked poultry or eating poultry outside the home, eating non-poultry meat outside the home, eating raw seafood, drinking raw milk, living on or visiting a farm and having contact with farm animals, contact with puppies were all risk factors for *Campylobacter* infection. Interestingly, eating poultry prepared in the home was associated with decreased odds of infection (Friedman, Neimann et al. 2000). Another study of sporadic cases of *Campylobacter jejuni* in humans in Hawaii, again, found increasing risk when poultry was consumed at commercial food establishments and the recent history of prescribed use of antimicrobial agents. However, consumption of chicken prepared in the home or eating beef products was inversely associated with illness. (Effler, Ieong et al. 2001)

The aforementioned papers illustrate the complex, often inconsistent, and poorly understood epidemiology of human *Campylobacter* cases and possible risk factors worldwide. There are several recurrent themes, however. Consumption of raw milk and contaminated water are consistently associated with both sporadic cases and outbreaks of campylobacterosis in humans. Since the source of raw milk is most frequently dairy cattle (with the exception of one goat milk outbreak (Harris, Kimball et al. 1987)) and cattle manure could be contributing fecal contamination to surface waters, the role of cattle in human infection warrants further investigation (Frost 2001)

The role of cattle in cases or outbreaks of human campylobacterosis

Ruminants such as cattle and sheep have also been identified as major reservoirs of this bacteria. The prevalence of *Campylobacter* isolated from cattle has ranged from 24% (Manser and Dalziel 1985), 37% (Wesley, Wells et al. 2000) 54% (Grau 1988), up to 79% (Atabay and Correy 1998). Shedding of *Campylobacter* has been associated with feed sources, age, and health status of animals. Pasture fed cattle shed less *Campylobacter* than cattle on feed in lot confinement (Grau 1988). Calves also were found to carry *Campylobacter* more frequently than adult cattle (Grau 1988). Prevalence of *Campylobacter* isolation from individual animals also varies significantly between herds (Atabay and Correy 1998). Since intestinal carriage is common in ruminants, human infection

presumably occurs through fecal contamination of milk, meat, or human contact with animal fecal material. *Campylobacter* have been isolated on cull cattle hides (Green, Kaneene et al. 2001) bulk tank raw milk (Beumer, Cruysen et al. 1988; Green, Kaneene et al. 2001). Therefore, it is not surprising that cattle have been identified as a source of this foodborne pathogen on numerous occasions

Several milkborne outbreaks of campylobacterosis have been described in detail. In Iowa unpasteurized milk and dairy cattle were investigated as a possible source of infection for 168 human cases reported to the Iowa Sate Health Department from August 1981 through July 1982 (Warner, Bryner et al. 1986). In surveying 477 dairy cattle, it was determined that 15.5% of animals carried *Campylobacter*. Serotyping of the human and cattle *Campylobacter* isolates, determined that 23% of the human cases were likely to come from a cattle source, rather than chickens, pigs, or sheep (Warner, Bryner et al. 1986). Interestingly, urban residents accounted for 75% of all of the *Campylobacter* cases; whereas, the 54% of the milk borne cases were rural residents (Warner, Bryner et al. 1986). Of the 168 milk borne infections, 50% were children less than 9 years of age. Unfortunately it was not reported as to how many of the human cases were outbreaks or the differences of exposures of these in comparison to sporadic human infection. Also, among rural cases, it was not defined which rural residents were dairy farm residents, who may have relatively endemic exposure to this organism in comparison to which cases did not live on

farms, who may have been naïve to *Campylobacter* exposure. (Warner, Bryner et al. 1986).

In 1983 a community outbreak of campylobacterosis in the United Kingdom was investigated (Hutchinson, Bolton et al. 1985). Between June 9, 1983 and July 4, 1983, 118 persons met the case definition for gastroenteritis. A bi-modal distribution of cases suggested that 24 later cases may have been secondary cases of campylobacterosis. Of these, 75 human stools were examined, finding 50 specimens to be positive for *C. jejuni*. Interestingly, 10 asymptomatic persons had stool samples which were also positive for *C jejuni*. Sixty-five households, all receiving milk from the same source, were investigated and it was determined that 41 of the 65 had household members were positive for *C. jejuni*. Although the dairy farm had no reported illness in its animals, 4 milk filters, one bulk tank milk sample, and 2 of 40 cows' milk samples were positive for *C. jejuni* over the course of the investigation. Isolates were biotyped using the Penner scheme. *Campylobacter* from the human isolates and the dairy were of the same biotype and serotype (Hutchinson, Bolton et al. 1985).

More recently, the potential hazards of educational farm visits was illustrated in Wales during 1994 (Evans, Roberts et al. 1996). Thirty-eight nursery school children accompanied by thirteen adults participated in a field trip to a dairy farm. Of the 38 children and of the 13 adults, 53% and 23%, respectively, developed gastroenteritis(Evans, Roberts et al. 1996). Cohort

analysis among those taking part in the field trip determined that illness was associated with the consumption of raw milk, rather than contact with the farm animals. The risk of illness also demonstrated a dose-response to the amount of raw milk consumed during the farm visit (Evans, Roberts et al. 1996). While *Campylobacter* was isolated from feces of 4 of the 120 dairy cattle, the biotypes and resistotypes from the animals differed from the *Campylobacter* isolated from the human cases. Three secondary cases were also documented during this outbreak (Evans, Roberts et al. 1996)

Pulsed-field gel electrophoresis (PFGE) was used to identify the source of *Campylobacter* in an Austrian youth center (Lehner, Schneck et al. 2000). In the fall of 1998, thirty-eight children of the 64 attendees showed signs consistent with campylobacterosis. Twenty-eight persons were positive for *Campylobacter* on fecal culture, including one healthy staff member of the camp (Lehner, Schneck et al. 2000). Twenty cows were used for milk at the youth center. Of the twenty cows, 5 were culture positive for *Campylobacter*. *Campylobacter* could not be isolated from the dairy's milk; however, the likelihood for being ill was most highly associated to the consumption of raw milk rather than other food items. PFGE patterns of both the human isolates and dairy cattle isolates demonstrated the same *SmaI* and *SalI* restriction patterns (Lehner, Schneck et al. 2000). Interestingly, human to human transmission also occurred during this outbreak as a camp employee who cleaned restroom facilities became ill without either consuming milk or having contact with the children (Lehner, Schneck et al. 2000).

Enteritis has also been traced to the handling of animals shedding *Campylobacter*. A dairy farmer was diagnosed with campylobacterosis following the acquisition of 2 newborn Holstein calves. Both calves displayed signs of septic arthritis and bloody diarrhea. Serotyping established the link between the farmer's gastroenteritis and the calves' diarrhea as both being caused by the sample serotype and biotype of *Campylobacter* (Dilworth, Lior et al. 1988)

In December of 2001, an outbreak of enteritis occurred in Wisconsin. This event illustrates the risk associated with the consumption of raw milk and misconceptions about perceived benefits of raw and 'natural' products by some consumers. In the Wisconsin outbreak, 75 persons met the case definition for enteritis. Of the 29 stool samples collected, 97% were culture positive for *C. jejuni*. The ages of cases ranged from 2 to 63 years of and culture positive stool samples also included mothers of case patients, who did not consume raw milk. This indicated secondary transfer from the ill children to their mothers, which is not commonly identified in *Campylobacter* cases. Pulsed-field gel electrophoresis (PFGE) confirmed identical strains in 21 isolates analyzed. The dairy's bulk tank milk was also culture positive for the identical PFGE pattern of *C jejuni*.

The Wisconsin outbreak also demonstrates intentional risky behavior of consuming raw milk in both young children and older adults. All of the cases

identified had consumed raw milk from a Grade A organic dairy farm which maintained a herd of 36 cows. Unpasteurized milk cannot be legally sold in Wisconsin. However, the dairy would provide raw milk during local events and distributed milk to consumers though a cow leasing program. Those interesting in circumventing the safety measures of buying pasteurized milk at retail stores could pay a fee to lease a share of the organic dairy herd. (CDC 2002)

In a study from the Netherlands of the prevalence of *Campylobacter* in cattle and milk, Beumer et al., found 22% of cattle samples positive and 4.5% of milk samples to contain *Campylobacter.* This study demonstrated that the lactoperoxidase present in milk can rapidly reduce counts of campylobacter, and that the inactivation of this enzyme resulted in higher recovery rates of *Campylobacter.* From the farms sampled, positive milk samples ranged from 0% of the farm's milk samples being positive to a farm with 10% of milk samples being culture positive for *Campylobacter.* Unfortunately this finding is not representative of potential human exposure since samples were taken at the receiving jar prior to filtration with the in-line milk filter which may reduce the pathogen load actually reaching the bulk tank. While the authors conclude that poor milking hygiene may contribute to fecal contamination of milk, they did not score the hygiene and udder preparation on each farm in order to assess the impact of milking parlor hygiene on *Campylobacter* recovery in milk (Beumer, Cruysen et al. 1988)

There has been an increasing consumer interest in raw and minimally processed foods as the outbreak in Wisconsin in 2001 demonstrates. These consumers believe that raw milk tastes better, provides greater nutrition, and may be protective for certain medical conditions. However, none of these claims or beliefs has been supported with any scientific evidence. (Potter, Kaufmann et al. 1984) Proponents of raw milk consumption also propose that it contains factors which enhance resistance to disease, enhance fertility, such as beneficial enzymes, hormones and antibodies. To the contrary, raw milk has been associated to disease such as campylobacterosis and salmonellosis in both cats and humans. Enzymes and hormones are either degraded by digestive enzymes upon consumption and are of no benefit to the human, and many peptides and antibodies are species specific factors which are not recognized as such by the human immune system. (Potter, Kaufmann et al. 1984)

Raw milk and other perceived "natural foods" have received increased consumer attention throughout the years. However, there is no established nutritional benefit of these products (Potter, Kaufmann et al. 1984). Unfortunately persons determined to obtain raw milk products will go to great length to circumvent safety measures in place to make the sale of raw milk illegal. Consequently concepts such as "cow-sharing or cow-leasing" have been developed (CDC 2002). There is ample evidence of the hazards of raw milk and

its products including *Salmonellosis, Listeria,* and *Campylobactosis* (Potter, Kaufmann et al. 1984); (CDC 2002).

Campylobacter can be isolated from red meats such as lamb, beef, and pork. However, the rate of recover of *Campylobacter* from beef products is very low, such that less than 5% of the beef samples may carry this bacteria (Harris, Thompson et al. 1986). A recent survey of retail meats in the United States failed to isolate any *Campylobacter* from ground beef (White, English et al. 2003). As described above in the case-control studies for human infection, consumption of red meats is rarely risk factors for campylobacterosis. Few individual cases of gastroenteritis and no outbreaks have been linked to beef products (Harris, Thompson et al. 1986; Kramer, Frost et al. 2000).

Another route of exposure of *Campylobacter* to humans has been through contaminated water sources. Outbreaks of Campylobacterosis have been acquired through consumption of water in Wales (Duke, Breathnach et al. 1996) England (Furtado, Adak et al. 1998) Sweden (Melby, Svendby et al. 2000) and Switzerland (Maurer and Sturchler 2000). A waterborne outbreak involving both E coli and *Campylobacter* occurred at a county fair in New York state during 1999 (CDC 1999). During any of the above mentioned waterborne outbreaks, tracing the initial source of the *Campylobacter* may be difficult to determine, since humans, wild and domestic animals may have the opportunity to contaminate the water source implicated. However, the possibility of agricultural run-off from

animal facilities cannot be excluded. Molecular typing often does not clarify the source of *Campylobacter* (Duke, Breathnach et al. 1996). The population genetics of this bacteria and its inherit genetic instability contribute to difficulty in identifying strain within or distinguishing strains between outbreaks (Meinersmann 2000).

Antimicrobial resistance in *Campylobacter* isolated from cattle

Another primary concern with *Campylobacter* is that this organism has demonstrated the ability to develop resistance to antimicrobial medications. Human isolates are displaying increased resistance to many classes of the drugs throughout time and introduction of new pharmaceuticals (Aarestrup, Nielsen et al. 1997; Engberg, Aarestrup et al. 2000). However, much of the work in this area has focused only on fluoroquinolones and the presumptive role of poultry in human infections (Smith, Besser et al. 1999; Nackamkin, Ung et al. 2002). Due to the role of cattle and risk factors described above, it would seem prudent to review antimicrobial resistance in *Campylobacter* isolated this food animal species.

In evaluating the literature for patterns of antimicrobial resistance in *Campylobacter*, it is helpful to compare 1) which drugs were tested 2) overall level of resistance 3) comparison between cattle and other populations included in the study, and 4) risk factors evaluated for the development of resistance.

Few researchers have evaluated dairy cattle and the farm environment for antimicrobial resistance in *Campylobacter.* Piddock and colleagues surveyed farm animals and environments in Lancashire UK including sheep, dairy cattle, wild birds, slurry and surface water. (Piddock, Ricci et al. 2000). Piddock and colleagues tested *Campylobacter* susceptibility to 5 antimicrobials including nalidixic acid, ciprofloxacin, erythromycin, tetracycline and kanamycin. They found that half of their 96 *C. jejuni* isolates from farm animals & environments were moderately resistant to erythromycin at 32 ug/ml. Thirty-five percent of isolates were resistant to Nalidixic acid at a concentration of 32 ug/ml. Tetracycline resistant isolates were classified as those with MIC > 8 ug/ ml and 25% of the *Campylobacter* were found to be resistant. While many isolates displayed intermediate resistant to ciprofloxacin with MICs 1-2 ug/ml, no isolates were highly resistant with MIC > 32 ug/ml. The most resistance was observed to kanamycin, since 70% of isolates required 8 ug/ml to inhibit growth. The most interesting finding was that no association could be found between the resistance of *Campylobacter* on a given farm and that farm's antibiotic use, nor could an association between individual animal treatments and resistance be established (Piddock, Ricci et al. 2000). Summary of resistance profiles for *Campylobacter* included isolates from 19 adult dairy cows, 11 calves, and five dairy farms (Piddock, Ricci et al. 2000). The data presented were insufficient in source and sample size for comparisons between animal source or herd. The majority of all isolates (51/ 96) including cattle, sheep, starlings, slurry, and the only calves

isolates were from the same farm, farm No 4. The other isolate sources included 6 other farms and refuse landfill (Piddock, Ricci et al. 2000)

Cabrita et al., in 1992, studied *Campylobacter* in wild & domestic animals in Portugal. This researched summarized resistance patterns to 7 antimicrobials including ampicillin, tetracycline, erythromycin, streptomycin, kanamycin, and gentamicin. Of the 183 isolates, resistance was 5.5 % to ampicillin, 5.5% to tetracycline, 12.6% to erythromycin, 23.5% to streptomycin, and 1.6% to kanamycin (Cabrita, Rodrigues et al. 1992).

The authors noted that tetracycline resistance was 6.2% in cattle, 5.1% in chicken, and 5.7% in swine. Erythromycin resistance was 6.2% in cattle, 5.1% in chicken, 26.2% in swine, and 3.7% in sheep. Streptomycin resistance was 15.6% in cattle, it was not noted in chicken, 58.4% in swine, and 11.1% in sheep. By comparison the overall resistance rates found in the study across all species sampled for ampicillin, tetracycline, erythromycin, streptomycin were 5.5%, 5.5%, 12.6% and 43%. It must be noted, however, that the above comparison in resistance rates primarily includes *C. coli* in swine, while other species were represented by *C. jejuni*. Plasmid carriage rates were also determined to be associated to streptomycin, tetracycline & erythromycin resistance. Highest rate of plasmid carriage was pigs > rats> chicken > cow isolates (Cabrita, Rodrigues et al. 1992). While these authors presented a descriptive work of resistance rates or plasmid carriage, no statistical comparisons across species or risk factor for their findings were made. Cattle isolates comprised 32 of the 183 isolates

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and all animals sampled were simply described as being from "healthy animals". Thus, it is not clear if these were on-farm or slaughter samples. The exposure of farm animals was assumed to be "antibiotics listed for use as feed supplements and veterinary therapeutics". However, they did not ascertain exposure of the animals sampled in the study nor describe differences in drug use by food animal species (Cabrita, Rodrigues et al. 1992).

The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) were established by the Danish Ministry of Food, Agriculture and Fisheries and the Danish Ministry of Health in 1995 (Bager, Aarestrup et al. 1999). This system has primary objectives of monitoring the usage of antimicrobial agents, tracking trends in occurrence of antimicrobial resistance and establish associations between use of antimicrobials in animals and humans to the observed resistance patterns in zoonotic, pathogenic and indicator bacteria (Bager, Aarestrup et al. 1999). The 2002 data across which species and antimicrobial resistance by drug can be compared found that among *C. jejuni* tetracycline resistance was 2% in broilers, 6% in cattle, and 15% in human isolates. Ampicillin resistance was 8% in broilers, 11% in cattle, however not listed for human isolates. Neomycin resistance was 0% in broilers, 2% in cattle, however not provided for humans. Streptomycin resistance was 0% in broilers, 2% in cattle, and 0% for human cases. Ciprofloxacin was 0% in broilers, 11% in cattle, and 17% in human isolates. Nalidixic Acid resistance was 0% in boilers, 11% in cattle, and 17% in human *C. jejuni*. Antimicrobials with 0%

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resistance demonstrated in either cattle or chicken *C. jejuni* include chloramphenicol, sulfonamide, erythromycin, and gentamicin (Emborg and Heuer 2002). The isolates included in the study are from animals at slaughter either representing a flock (n=53 broilers) or a herd (n= 53 cattle). Human isolates are submitted from diagnostic laboratories and represent 93 domestically acquired cases of *C. jejuni* gastroenteritis. Interestingly, isolates that are from cases of Campylobacterosis which are acquired during travel outside of Denmark demonstrated much higher levels of resistance to tetracycline, gentamicin, streptomycin, and ciprofloxacin of 42%, 5%, 5%, and 79%, respectively.

Thus far the determination of statistical association with resistance patterns across the species (besides foreign travel) has not been presented as part of a DANMAP report (Emborg and Heuer 2002). Extensive descriptive data are presented on antimicrobials used in food animal species and human consumption across Denmark; however, the exposure of each chicken flock or cattle herd representing the *Campylobacter* isolates is not described (Emborg and Heuer 2002). Also, the distributions of dairy and beef cattle which make up the slaughter samples is not described. It is interesting to note that domestically acquired human infections with *Campylobacter* in Denmark demonstrate 17% resistance to fluoroquinolones, although the poultry flock population is 100% susceptible to this drug class. Fluoroquinolones are used in poultry production in Denmark including broilers flocks (Emborg and Heuer 2002). Associations between food animal use and resistance patterns are not clear even in this very

comprehensive surveillance program and risk factors for resistant human infections required further exploration.

Patterns of antimicrobial resistance were recently described in *Campylobacter spp.* in Germany across several food animal types at slaughter including broilers (n=58), pigs (n=51), cattle (n=34) and human clinical isolates (n=37) (Bartelt, Vogt et al. 2003). This study found erythromycin resistance to be 37.3% in pigs, 2.9% in cattle, 0% in boilers, and 10.8% in humans. Ampicillin resistance was 3.9% in pigs, 2.9% in cattle, 37.9% in broilers, and 10.8% in human isolates. Nalidixic acid resistant was 13.7% in pigs, 11.8% in cattle 55.2% in broilers, and 5.4% in human isolates. Ciprofloxacin resistance was 13.7% in pig, 5.9% in cattle , 55.2% in broilers, and 5.4 % in humans. Tetracycline resistance was 60.8% in pigs, 35.3% in cattle, 29.3% in broilers, and 13.5% in humans. Gentamicin resistance was not present in *Campylobacter* from any source (Bartelt, Vogt et al. 2003).

These authors did assess associations for *Campylobacter* resistance by animal source. Interestingly, the animal associations to nalidixic acid and ciprofloxacin are not the same. Ciprofloxacin resistance was significantly lower in both cattle and human isolates, and significantly higher in broiler isolates ($p < 0.01$). Whereas, the level of resistance to nalidixic acid was only significantly lower in human isolates, but still was significantly higher in boiler isolates($p <$

0.01). The resistance level to ampicillin was significantly lower in swine isolates and significantly higher in broilers ($p < 0.01$). The resistance level was significantly higher in pigs and significantly lower in poultry for erythromycin ($p < 0.01$). The level of tetracycline resistance was significantly higher in pigs and significantly lower in humans ($p < 0.01$) (Bartelt, Vogt et al. 2003). Most of the *Campylobacter* isolated from pigs was *C. coli*, whereas *C jejuni* was more frequently isolated from human, broilers and cattle (Bartelt, Vogt et al. 2003). Antimicrobial use in food animals in Germany was not described in this study (Bartelt, Vogt et al. 2003). This study also illustrated that the patterns of antimicrobial resistance demonstrated by human isolates does not necessarily follow any food animal species, including chicken. Unlike the Danish surveillance described above, human *Campylobacter* isolates in Germany are less resistant than potential food animal sources. Clearly further study of risk factors for human resistance in *Campylobacter* is required.

A survey of antibiotic resistance was performed from *Campylobacter* isolated from farmland in the United Kingdom (Leatherbarrow, Williams et al. 2003). Farmland included in this study was primarily considered mixed dairy farms. The authors sampled cattle (n = 1014), water (n=137), birds (n=180), sheep (n=24), wildlife (n=271), and soil (1015). Antimicrobial resistance was summarized across *Campylobacter* type, but not by sample source. *C. coli* (n=81), which was isolated mostly from water and sheep was 7% resistant to erythromycin and susceptible to nalidixic acid, ciprofloxacin, ampicillin and

augmentin (Leatherbarrow, Williams et al. 2003). *C jejuni* (n=427) which included cattle, water, bird, sheep, and wildlife isolates was 1.6% resistant to nalidixic acid, 1.1% resistant to ciprofloxacin, 18.7 % resistant to erythromycin, and 6.8% resistant to ampicillin. No resistance to augmentin was demonstrated in *C. jejuni* isolates (Leatherbarrow, Williams et al. 2003). No on-farm use of antimicrobials was described and antimicrobial resistance by isolate source was not distinguished (Leatherbarrow, Williams et al. 2003). Authors did demonstrate similarity in *Campylobacter* strains by PFGE in which 80% of cattle, 6% of wildlife, and 6% of water were closely genetically associated in one dendogram cluster. Another dendogram cluster contained 37% of bird, 29% of wildlife and 29% of water isolates (Leatherbarrow, Williams et al. 2003). Understanding the relatedness of *Campylobacter* isolates through genetic typing will further the study of resistance determinants and their genetic exchange.

Conclusions

Cattle have been shown to be reservoirs for *Campylobacter spp,* particularly *C jejuni.* The first objective was to summarize risk factors for human cases of campylobacterosis. Case-control evaluation of risk factors for infection was most frequently used; however, inconsistencies in the findings across research teams are evident. Human infections have been associated to dairy products or animal exposure.

Little research has focused on risk factors for antimicrobial resistance in *Campylobacter* isolated on dairy farms or from animals. Therefore, generalized

slaughter isolates from cattle may better represent risk factors for cattle intentionally raised for beef, which differs from management style and antimicrobial practices allowed on dairy farms. While pattern of resistance in *Campylobacter* do vary across species within studies, few associations between actual animal exposure to antimicrobials have been assessed by researchers. Those researchers which have identified on-farm or individual animal use of drugs, did not find that antimicrobial use was related to observed resistance patterns. It is also clear that antimicrobial resistance patterns in *Campylobacter* isolated from humans require further analysis to identify risk factors and that role of dairy cattle should be part of such an assessment. This understanding will facilitate the means to thoughtfully mitigate the dissemination potentially untreatable infections which cattle may transmit to humans either through contact or consumption of food products.

The second objective involved case discussion of outbreaks of human Campylobacter directly linked to dairy cattle, either through contact or consumption of dairy products. Consumption of raw milk or contact with farms animals are frequently the point of exposure, although the role of dairy animals in contamination of surface water warrants further study. The third objective was to describe patterns of antimicrobial resistance in Campylobacter isolated from cattle as well as the differing. It was found that much disparity in laboratory techniques and antimicrobials studied by each research teams makes direct comparisons not feasible.

CHAPTER TWO

Patterns of antimicrobial susceptibility in *Campylobacter* isolated from organic and conventional dairy farms in the Midwestern and Northeastern United States

Structured Abstract

Objective: To describe patterns of antimicrobial susceptibility in *Campylobacter* isolated from organic and conventional dairy farms in the Midwest and Northeast U.S.

Design: Longitudinal study.

Sample Population: Antimicrobial susceptibility was performed on 2017 *Campylobacter* isolates from 128 farms in Michigan, Minnesota, New York and Wisconsin. Results consist of 458 *Campylobacter* isolates from organic farms and 1559 isolates from conventional dairies.

Procedure: Sampling and data collection occurred every two months from August 2000 to October 2001. Fecal samples were collected from healthy cows, calves and other targeted cattle groups and from bulk tank milk, milk filters, water, feed sources, and cattle housing. *Campylobacter* identification and antimicrobial susceptibility was performed at a central laboratory, at Michigan State University.

Results: Most isolates (> 97%) from both farm types were susceptible to amoxi-clav, azithromycin, ceftriaxone, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, florfenicol, gentamicin, nalidixic acid, and streptomycin. Isolates from either farm type appeared to be intrinsically resistant (>97.5%) to ceftiofur,

cephalothin, and trimeth-sulfa. Varying levels of resistance were observed to ampicillin 8.6 and 7.1%, kanamycin 32.4 and 30.0, sulfamethoxazole 37.2 and 38.7% and tetracycline 58.3 and 49.3% of conventional and organic isolates, respectively. *Campylobacter* isolates from conventional dairy farms were statistically significantly more resistance to tetracycline ($p < 0.01$).

Conclusions and Clinical Relevance: *Campylobacter* from organic and conventional dairy farms has similar patterns of resistance.

Introduction

Campylobacter spp. is the most frequently identified cause of bacterial gastroenteritis in the United States (Acheson 2001) (Altekruse and Tollefson 2003). *Campylobacter* outnumbers other infectious causes of foodborne illness in the Unites States, such as Salmonella, E. coli O157:h7, and Shigella (Mead, Slutsker et al. 1999). Based on these data, each year 2 million cases of illness were estimated to be caused each year by this organism (Allos 2001). Most *Campylobacter* enteritis cases are mild, self limiting episodes of vomiting, cramping, and diarrhea (Tauxe, Hargrett-Bean et al. 1988) (Altekruse, Swerdlow et al. 1998). A more serious form of campylobacterosis can occur in infants, geriatric patients, and immune compromised individuals. In these cases bloody stools, dehydration, septicemia, and long-term sequela can occur (Blaser 1997). Secondary effects of *Campylobacter* gastroenteritis can include the demyelinating neurologic disorder Guillian-Barre syndrome (GBS) or intermittent arthritis (Rees, Soudain et al. 1995) (Nachamkin, Allos et al. 1998). The former

occurs subsequent to about 1 in 1000 cases of *Campylobacter* enteritis. Guillian-Barre syndrome is usually transient, but some GBS sufferers continue to have neurologic deficits throughout life (Rees, Soudain et al. 1995).

Thermophilic *Campylobacter* can colonize the gastrointestinal tracts of mammals and birds without causing disease (Manser and Dalziel 1985). Thus, feces from normally appearing animals may contaminate the environment with *Campylobacter* organisms. Consequently, many human infections are associated with direct or indirect animal exposure (Deming, Tauxe et al. 1987). Research has already focused on the role of *Campylobacter*-contaminated poultry in retail markets (Harris, Thompson et al. 1986; Jacob-Reitsma, Koenraad et al. 1994) (Smith, Besser et al. 1999; Nackamkin, Ung et al. 2002). However, the dairy industry may also be a source of human exposure to *Campylobacter* organisms. It has already been established that healthy adult cows and calves frequently shed this organism in their manure (Green, Kaneene et al. 2001) (Wesley, Wells et al. 2000) (Nielsen 2002). Moreover, a number of outbreaks of *Campylobacter* enteritis have been associated with raw milk consumption (Warner, Bryner et al. 1986) (Dilworth, Lior et al. 1988) (Kalman, Szollosi et al. 2000) (Lehner, Schneck et al. 2000), dairy farm visits (Evans, Roberts et al. 1996), and water contamination (Duke, Breathnach et al. 1996) (Melby, Svendby et al. 2000) (Frost, Gillespie et al. 2002) . Therefore, the dairy industry must be examined for the role it may play in contributing this foodborne pathogen to human food and water sources.

Another primary concern with *Campylobacter* is that this organism has demonstrated the ability to develop resistance to antimicrobial agents. *Campylobacter* isolates from humans are displaying increased resistance to many classes of the drugs throughout time and with the introduction of new pharmaceuticals (Neu 1992) (Engberg, Aarestrup et al. 2000). Increasing antimicrobial resistance is a global problem. In developing countries, antimicrobial resistance is highly correlated to lax restrictions on the use of these drugs and easy access to by humans to pharmaceuticals (Blaser 1997). This results in self-medicating to compensate for poor sanitary conditions (Oberhelman and Taylor 2000) (Padungtod and Kaneene 2003) In developed countries, there is ongoing debate regarding the contribution of human medical, veterinary therapeutic and animal husbandry practices to the decreased susceptibility of key bacteria to antimicrobials (VanDenBogaard 1997; Smith, Bender et al. 2000; Threlfall, Ward et al. 2000; Wagner, Jabbusch et al. 2003). There is documentation of increased fluoroquinolone resistance in *Campylobacter* and other bacteria once these antimicrobials were approved in some food animal species(Smith, Besser et al. 1999; McDermott, Bodeis et al. 2002). Also, there has been evidence of increased susceptibility in bacteria when certain antimicrobials were banned from use (Aarestrup, Seyfarth et al. 2001) (Boerlin, Wissing et al. 2001). However, most studies supporting the decrease in susceptibility are based on ecological (aggregative) analysis of data (i.e. which drugs are approved for veterinary use in a particular country) without ascertaining actual exposure to the drugs being studied. Also the focus of much

research on *Campylobacter* resistance has been on drug classes such as fluoroquinolones and macrolides, while the antimicrobials used on dairy farms are limited. (Hady, Lloyd et al. 1993; Sundlof, Kaneene et al. 1995) Thus, the role of dairy farm practices to the development of antimicrobial resistance in *Campylobacter* remains poorly defined despite numerous outbreaks of enteritis that have been directly associated with dairy sources.

Therefore, the objective of this study is to describe the antimicrobial susceptibility of *Campylobacter* isolates obtained from organic and conventional dairy farms across key animal management groups.

Materials and Methods

Herds: 132 dairy farms were selected from four states: Michigan, Minnesota, New York, and Wisconsin. Data are reported on 128 farms from which *Campylobacter* isolates were available for antimicrobial testing. . Herds were enrolled according to farm type (organic vs. conventional) and by farm size (number of cows, both milking and dry). To be included in the study, a herd had to meet the following criteria: 1) at least 30 milking cows, 2) at least 90% of cows of Holstein breed, 3) raise their own calves for replacement cattle, and 4) ship milk all year. Organic farms had to be certified as organic by a recognized organic certification agency and may not have used antimicrobials in cattle greater than 1 year of age for at least 3 years. For conventional farms, lists of farms were obtained from the respective State Departments of Agriculture, and herds within approximately 100 miles of the respective universities were

randomly selected to receive a mailing describing the research project. Farms were asked to indicate interest in participation by returning a postcard. The final list of farms was obtained by randomly selecting names of respondents that had indicated willingness to participate. In order to evaluate potential herd management practices as risk factors, a predetermined numbers of farms were enrolled within the following size categories (by number of cows, both milking and dry) of 30-49, 50-99, 100-199, 200 & up. Due to limited availability of organic farms, all known organic farms within approximately 150 miles of the respective universities were contacted to determine eligibility based on the selection criteria and their desire to participate.

Cattle samples: Cattle samples were collected by placing approximately ten grams of fecal material obtained by rectal retrieval into Whirl-Pak® bags. A separate glove was used for the collection of each sample. The number of samples collected per herd and the number collected from specific cattle groups was based on the herd size. The total number of animal samples from herds with 30-49, 50-99, 100-199, and ≥ 200 cows was 30, 40, 50, and 55 animal samples, respectively. Cattle management classifications included pre-weaned heifer calves, cows to be culled within 14 days, periparturient cows (due to calve within 14 days and cows within 14 days in milk after calving), cows designated as "sick" by farm personnel or herd veterinarian, and healthy lactating cows. No effort was made to collect samples from the same cattle at subsequent herd visits.

Environmental samples: One sample from each of the following locations was collected at each sampling visit by wiping areas to be tested with sterile gauze pads soaked in double strength skim milk: maternity pen, sick pen, calf housing, feedbunk of the lactating cows, lagoon or manure pile, and bird droppings. A sample from cattle water source (a water tank or a pooled swab from five drinking cups), a bulk tank milk sample, and a milk line filter were also collected. If a cow was designated to be culled, the haircoat across the lower flank and rump was swabbed. If a pen location was not used on a particular farm (e.g., no sick pen) then no sample was collected for that location. If there was shared use of some facilities such as with the sick cow pen and calving pen, the sample was labeled according to the predominant use.

Shipment: After collection, samples were shipped to a central laboratory at Michigan State University. Samples from Minnesota, New York and Wisconsin were shipped via overnight delivery in Styrofoam boxes with ice packs. Samples were shipped the same day as collection whenever possible; however, some samples were stored in a refrigerator for 12-36 hours until the next shipping opportunity.

***Campylobacter spp.* Isolation and Identification:** Environmental swabs and milk filters were enriched in Bolton broth (Oxoid) containing 5% laked horse blood and selective antimicrobial agents (20mg/L cefaperazone, 20 mg/L vancomycin, 20 mg/L trimethoprim, 50 mg/L cycloheximide). The enriched samples were then incubated at 42° C in 5-10% CO_2 for 48 hours. Animal fecal samples and milk samples were suspended in phosphate buffer saline (PBS)

solution. The PBS suspended biological samples and enriched samples were streaked on selective *Campylobacter* Blaser plates (BD Diagnostics,) and incubated at 42° C in 5-10% CO_2 for 48 hours. Typical colonies (small pinpoint gray colonies without hemolysis) were selected and streaked on sheep blood agar (SBA) and incubated at 42°C in 5-10% CO_2 for 48 hours. *Campylobacter* identification was performed from isolated colonies by gram staining, oxidase testing, and motility testing. Hippurate hydrolysis was used to speciate *C. jejuni* using ATCC 33560 as a positive control and *C. coli* as a negative control.

In vitro susceptibility testing –Microbroth Dilution: *In vitro* susceptibility testing was performed using the microbroth dilution method, following guidelines provided by the National Committee on Clinical Laboratory Standards (NCCLS) (NCCLS 2003). Bacterial isolates from frozen stock were grown on Brucella agar supplemented with 5% defribrinated sheep blood (BASB) for 48 hours at 42°C under microaerophilic conditions. Individual colonies from each plate were subcultured on BASB under similar growth conditions. Bacteria were swabbed from the BASB and suspended in 5 ml H_2O and the turbidity was adjusted to a 0.5 McFarland standard. This suspension was used to make a 1:10 dilution into Haemophilus testing medium (HTM), resulting in a final bacterial inoculum concentration of approximately 8 x 10^5 CFU/ml.

Customized microbroth dilution plates (CMV1USDA) were purchased pre-made from TREK Diagnostic Systems, Inc., with a prepared range of drug concentrations of azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, and tetracycline (Table 1). *C. jejuni*

ATCC33560 and 81176 were used as quality control strains. Each plate was inoculated by adding 100 ul of the bacterial suspension using a Sensititre autoinoculator, covered with a gas-permeable seal, and incubated at 42°C in microaerophilic conditions for 48 hours. The minimum inhibitory concentration (MIC) was determined as the minimum antimicrobial dilution at which no bacterial growth occurred. Following the observation that dairy isolates did not demonstrate resistance patterns similar to humans, another customized antimicrobial panel (CMV2DMSU) was developed with Trek Diagnostics to address drug exposures that are common to dairy cattle management and may allow comparison for animals co-infected with Salmonella. This antimicrobial panel included 17 drugs encompassing drug classes used on our study farms such as beta lactams & cephalosporins (Geiger, Ruegg et al. 2003). The breakpoints used to categorize isolates as resistant or not resistant were those recommended by the National Antimicrobial Resistance Monitoring System (NARMS) for *Campylobacter* for azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, tetracycline (Table 1). For the expanded 17 drug panel, general enteric breakpoints were used to classify isolates as resistant for the additional antimicrobials (Table 1).

Table 1: Dilution ranges for the antimicrobial agents used and interpretative breakpoints

Antimicrobial	CMV1USDA panel format (ug/ml)	CMV2DMSU panel format (ug/ml)	Interpretative Criteria For Resistant Strains (ug/ml)
Amoxicillin-Clavulanic Acid	N/A	2/1 - 64/32 (Amox/Clav)	≥ 32/16 [A]
Ampicillin	N/A	2 - 64	≥ 32 [A]
Azithromycin	0.03 - 256	0.12 - 4	≥ 2 [B]
Ceftiofur	N/A	1 - 16	≥ 8 [A]
Ceftriaxone	N/A	4 - 128	≥ 64 [A]
Cephalothin	N/A	4 - 64	≥ 32 [A]
Chloramphenicol	0.5 - 64	4 - 64	≥ 32 [A]
Ciprofloxacin	0.03 - 64	0.5 - 16	≥ 4 [B]
Clindamycin	0.06 - 256	N/A	≥ 4 [B]
Erythromycin	0.12 - 256	0.25 - 16	≥ 8 [B]
Florfenicol	N/A	2 - 32	≥ 16 [C]
Gentamicin	0.12 - 256	2 - 32	≥ 16 [B]
Kanamycin	N/A	8 - 128	≥ 64 [A]
Nalidixic Acid	0.12 - 128	4 - 128	≥ 32 [B]
Streptomycin	N/A	16 - 128	≥ 64 [A]
Sulfamethoxazole	N/A	64 - 512	≥ 512 [A]
Tetracycline	0.25 - 256	2 - 128	≥ 16 [B]
Trimethoprim Sulfamethoxazole	N/A	1/19 - 8/512 (Trimeth/Sulf)	≥ 4/76 [A] (Trimeth/Sulfa)

[A] General Enteric Breakpoint

[B] *Campylobacter* Breakpoint used by NARMS

[C] Gram Negative Veterinary Diagnostic Breakpoint

Data analysis: To determine if there was an association with the level of resistance and farm type, descriptive breakpoints were used to classify isolates as resistant or susceptible for each antimicrobial agent. The proportion of resistant isolates by herd type (organic or conventional) were analyzed using Chi-square tests with SAS version 8.2 (Cary, North Carolina).

Results

Data have been summarized for the antimicrobial susceptibility testing of 2017 *Campylobacter* isolates. This summary includes isolates which represent 128 farms. Isolates from 450 animals on organic farms were tested, while 8 environmental *Campylobacter* isolates were available for antimicrobial testing from organic farms. Isolates from 1526 animals on conventional farms were tested, and 33 environmental samples from conventional farms were tested for antimicrobial susceptibility (Table 2).

Table 2. Distribution of Isolates used for antimicrobial testing from different sources by farm type

	Organic Farm	Conventional Farm
Environmental Isolates		
Feedbunk	0	0
Calf pen	2	1
Sick cow pen	0	1
Maternity pen	2	3
Water tank	1	3
Lagoon	1	3
Bulk tank milk	0	3
Milk filter	2	9
Bird droppings	0	4
Cull cow haircoat	0	6
Total Environmental Isolates	**8**	**33**
Cattle Isolates		
Pre-weaned calves	132	427
Healthy lactating	238	683
Cull cows	3	32
Pre-fresh cows	23	80
Fresh cows	35	177
Sick cows	19	126
Total Cattle Isolates	**450**	**1526**
Total Isolates	458	1559

Over 97% of our isolates were classified as *C. jejuni* (Green, Kaneene et al. 2001). There has been a recent convention to summarize the dispersion of antimicrobial susceptibilities by MIC_{50} and MIC_{90} to describe the antimicrobial concentration of each drug which inhibits 50% and 90% of the isolates respectively from a given source. However, as demonstrated in Table 3, this

information does not always capture differences that may be reflected in the proportion of resistant isolates. Also, guidelines which might be used to determine if a significant difference or change in MIC_{50} and MIC_{90} over time have not been established.

Across herd type, it was observed that conventional farms appear to have slightly more isolates resistant to ampicillin (8.6% vs. 7.1%), even though this difference was not statistically significant (p=0.52). Similarly, both the observed MIC_{50} and MIC_{90} were one dilution higher for organic farms than conventional farm isolates. For ceftriaxone, both the MIC_{90} and proportion of resistant isolates was higher for organic farm isolates than conventional isolates (2.3 % vs. 1.4%), even though this difference was not statistically significant (p =0.39). Ciprofloxacin resistance was slightly higher in conventional farm isolates compared to organic (1.1% vs. 0.9%), even though the MIC_{50} and MIC_{90} were identical. The other drug of choice for treatment of human campylobacterosis, erythromycin, also demonstrated similar resistance, MIC_{50}, and MIC_{90} across both herd types. Kanamycin resistance was fairly common in both herd types with 32.4% of conventional farm isolates and 30.0% of organic isolates demonstrating resistance. However, the MIC_{50} and MIC_{90} were identical for kanamycin in both herd types and the proportion of resistant isolates was not significantly different by herd type (p=0.56). Tetracycline resistance was common to both farm types. However, both the proportion of resistant isolates and MIC_{50} were significantly higher for conventional farm isolates when compared to the susceptibility of organic farm isolates (p=0.007). Conventional

farm isolates required four times the antimicrobial concentration of tetracycline (32 ug/ml) to inhibit growth of 50% of the isolates, while organic farm isolates required 8 ug/ml. The proportion of tetracycline resistant isolates was significantly higher for conventional farms 58.3% compared to tetracycline resistant isolates on organic farms 49.3% ($p=0.007$)

Table 3. Antimicrobial Susceptibility of *Campylobacter* isolated from cattle by Farm Type

Antimicrobial	FarmType[1]	Number of Isolates	MIC $_{50}$	MIC $_{90}$	% Resistant
Amoxicillin-Clav	C	686	2	2	0.1 %
	O	168	2	2	0.0 %
Ampicillin	C	686	4	8	8.6 %
	O	168	8	16	7.1 %
Azithromycin	C	1526	0.12	0.12	1.3 %
	O	450	0.06	0.12	1.1 %
Ceftiofur	C	686	16	16	97.7 %
	O	168	16	16	98.2 %
Ceftriaxone	C	686	16	16	1.4 %
	O	168	16	32	2.3 %
Cephalothin	C	686	64	64	99.3 %
	O	450	64	64	100 %
Chloramphenicol	C	1526	2	4	1.1 %
	O	450	2	4	0.0 %
Ciprofloxacin	C	1526	0.12	0.5	1.1 %
	O	450	0.12	0.5	0.9 %
Clindamycin	C	840	0.12	0.5	1.3 %
	O	282	0.12	0.25	1.0 %
Erythromycin	C	1526	0.5	1.0	1.2 %
	O	450	0.5	1.0	1.1 %
Florfenicol	C	686	2	2	0.3 %
	O	168	2	2	0.0 %
Gentamicin	C	1526	2	2	0.1 %
	O	450	1	2	0.0 %
Kanamycin	C	686	8	128	32.4 %
	O	168	8	128	30.0 %
Nalidixic Acid	C	1526	4	8	1.9 %
	O	450	4	8	1.3 %
Streptomycin	C	686	16	16	1.6 %
	O	168	16	16	0.6 %
Sulfamethoxazole	C	686	256	512	37.2 %
	O	168	256	256	38.7 %
Tetracycline	C	1526	32	128	58.3 %
	O	450	8	128	49.3 %
Trimethoprim Sulfamethoxazole	C	686	8	8	98.4 %
	O	168	8	8	98.8 %

Since *Campylobacter* recovery was very low in environmental samples (Green, Kaneene et al. 2001), susceptibility data are presented as the isolate distributions across the antimicrobial concentration ranges for each drug tested (Table 4). From our study we noted that overall resistance in the environmental isolates was low, even though higher MICs were observed by one conventional isolate to each ampicillin and eythromycin, while both organic & conventional isolates demonstrated higher MICs to tetracycline and sulfamethoxazole. No environmental isolates demonstrated any MIC that was above the breakpoint of 64 ug/ml to kanamycin.

Due to the increased consumer interest in raw milk and minimally processed food products, it is noteworthy that decreased susceptibility was observed in some raw milk and milk filter samples to the 8 antimicrobials of interest in treating human infections (Table 5). Decreased susceptibility was noted to nalidixic acid in 1 of 2 organic isolates tested from milk sources, while 7 of 12 *Campylobacter* isolates from milk and milk filters from conventional farms demonstrated decreased susceptibility to tetracycline.

An antibiogram was constructed for the 8 drugs surveyed under NARMS. This demonstrated that only a minority of either conventional or organic isolates were resistant to 2 or more antimicrobials, 3.1% and 1.5% respectively (Table 6, Figure 1). However when an antibiogram was constructed for the customized 17-drug panel, a higher proportion of multi-drug resistance was observed in both the organic and conventional isolates, 40% and 46.8% respectively (Table 7, Figure 2).

Table 4. Distribution of MIC for Environmental Isolates by Farm Type

MIC ug/ml																	
		.03	.06	.12	.25	.50	1	2	4	8	16	32	64	128	256	512
Drug	Type																
Amox Clav	C							9	1								
	O							1									
Amp	C							3	1	4	1		1				
	O							1									
Azith	C	10	4	15	3					1							
	O	3	3	2													
Cefti	C									2	8						
	O										1						
Ceftrx	C								1	1	6	2					
	O										1						
Ceph	C												10				
	O												1				
Chlor	C					1	8	13	1	1							
									0								
	O					1	3	3	1								
Cipro	C	1	6	13	1	11		1									
	O		3	3	1	1											
Clind	C		4	3	9	6							1				
	O		4	2	1	1											
Eythr	C			1	10	11	8	2				1					
	O				3	3	2										
Florfl	C							10									
	O							1									
Gent	C						1	14		1							
							8										
	O				1	1	3	3									
Kan	C									10							
	O									1							
Naldx	C							2	2	5		1					
									5								
	O							1	3	3		1					
Strept	C										10						
	O										1						
Sulfa	C												1	1	3	5	
	O															1	
Tetr	C				12			6		1	3	2	2	6	1		
	O				5	1		1					1				
Trimet Sulfa	C									10							
	O									1							

[1] Farm type C= conventional dairy isolates, O=organic dairy isolates
[2]Trek Diagnostics Custom antimicrobial panel CVM1USDA
(n=22 Conventional dairy isolates, n=7 Organic dairy isolates)
[3]Trek Diagnostics Custom antimicrobial panel CVM2DMSU
(n=10 Conventional dairy isolates, n=1 Organic dairy isolate)

Table 5. Distribution of Antimicrobial susceptibility in Isolates from Milk & Milk Filters

MIC ug/ml[2] Drug	Farm Type[1]	.03	.06	.12	.25	.50	1	2	4	8	16	32	64	128
Azith	C	5	2	3	2									
	O	1		1										
Chlor	C						6	4	2					
	O							2						
Cipro	C	1	3	6		2								
	O			1	1									
Clind	C		2	1	5	2								
	O		1		1									
Eyth	C			1	6	2	2	1						
	O					1	1							
Gent	C						8	3						
	O						1	1						
Naldx	C							1	9	2				
	O									1		1		
Tet	C				5			1		1	1	1	1	2
	O				1	1								

[1] Farm type C= conventional dairy isolates, O=organic dairy isolates

[2]Trek Diagnostics Custom antimicrobial panel CVM1USDA
(n=12 Conventional dairy isolates, n=2 Organic dairy isolates)

Table 6. Antibiogram of Resistance patterns (NARMS 8 drug panel)

Resistance Pattern Antimicrobial	Farm Type[1]	Number of isolates	Percentage of isolates
Susceptible to all	C	338	39.1%
Drugs tested	O	144	49.8%
Tet	C	493	57.1 %
	O	136	47.0 %
Ciprofloxacin	C	1	0.1 %
	O	1	0.3 %
Clindamycin	C	1	0.1 %
	O	0	
Eythromycin	C	0	
	O	0	
Gentamicin	C	0	
	O	0	
Nalidixic Acid	C	4	0.5 %
	O	4	1.4 %
Azi-Cip- Clind-Eryth-Nal-Tet	C	0	
	O	3	1.0 %
Azi- Clind-Eryth-Tet	C	7	0.8 %
	O	0	
Azi-Chlor-Cip- Clind-Eryth-Nal-Tet	C	1	0.1 %
	O	0	
Cip- Nal-Tet	C	7	0.8 %
	O	0	
Nal- Tet	C	5	0.6 %
	O	0	
Cip-Tet	C	1	0.1 %
	O	0	
Azi- Eryth	C	0	
	O	1	0.3 %
Azi- Clind-Eryth	C	2	0.2 %
	O	0	
Azi- Clind-Eryth-Nal	C	1	0.1 %
	O	0	
Azi- Clind-Cip-Eryth-Nal	C	1	0.1 %
	O	0	

Conventional isolates n=863

Organic isolates = 289

Table 7. Antibiogram of Resistance patterns (17 drug panel)

Resistance Pattern Antimicrobial	Farm Type[1]	Number of isolates	Percentage of isolates
Susceptible to all	C	177	25.4 %
Drugs tested	O	69	40.8 %
Kan	C	1	0.1 %
	O		
Amp	C	6	0.8 %
	O	2	1.2 %
Amp-Nal	C	1	0.1 %
	O		
Amp-SulfTtet	C	0	
	O	3	1.8 %
Cefx	C	1	0.1 %
	O		
Azith	C	1	0.1 %
	O		
Sulfa	C	99	14.2 %
	O	17	10.1 %
Amp-Sulfa	C	10	1.4 %
	O	1	0.1 %
Cefx-Sulfa	C	4	0.6 %
	O		
Azith-Eryth-Kan	C	1	0.1 %
	O		
Azith-Eryth-Strep	C		
	O	1	0.6 %
Amox-Amp-Azith-Chlor-Eryth-Flor-Kan-Nal-Strep	C	1	0.1 %
	O		
Chlor-Kan-Sulfa	C	1	0.1 %
	O		
Amp-Azith-Chlor-Eryth-Flor-Nal-Strep-Sulfa	C	1	
	O		
Amp-Cefx-Sulfa	C	1	0.1 %
	O		
Amp-Cefx-Sulfa - Tet	C	0	
	O	1	0.6 %
Kan-Sulfa	C	1	0.1 %
	O	1	0.6 %
Tet	C	86	12.4 %
	O	14	8.3 %
Kan-Tet	C	135	19.4 %
	O	29	17.2 %
Sulfa-Tet	C	54	7.8 %
	O	7	4.1 %
Kan-Sulfa-Tet	C	60	8.6 %
	O	17	10.1 %
Amp-Kan-Tet	C	11	1.6 %
	O	1	0.6 %
Amp-Tet	C	12	1.7 %
	O	4	2.4 %

Table 7 (cont'd)

Kan-Strep-Sulfa-Tet	C	3	0.4 %
	O		
Cip-Kan-Nal-Sulf-Tet	C	2	0.3 %
	O		
Ceftx-Sulfa-Tet	C	2	0.3 %
	O	1	0.6 %
Ceftx-Kan-Tet	C	1	0.1 %
	O	1	
Ceftx-Kan-Sulfa Tet	C		
	O	1	0.6 %

Conventional isolates (n=696)

Organic isolates (n=169)

Figure 1.

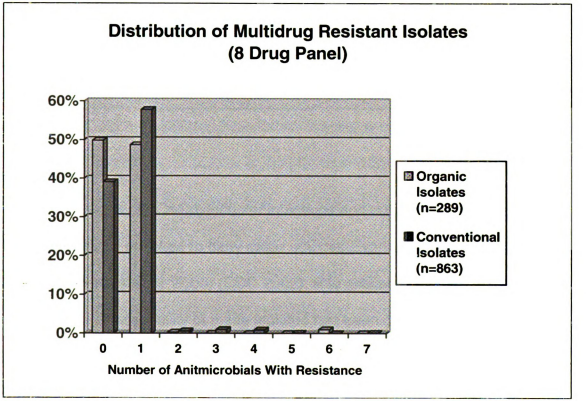
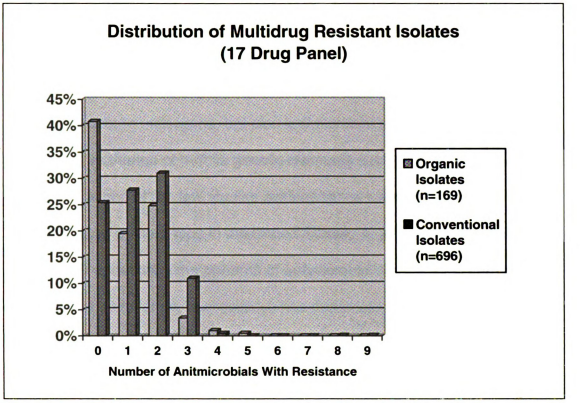


Figure 2.



Discussion

Although both *Campylobacter* infections and outbreaks in humans have been associated or linked to dairy cattle sources(Evans, Roberts et al. 1996) (Lehner, Schneck et al. 2000) (CDC 2002), little critical evaluation of the antimicrobial susceptibility of these isolates has been done. Since multi-drug resistant Salmonella infections in humans have been traced back to dairy farms through either meat or milk consumption(Spika, Waterman et al. 1987) (Villar, Macek et al. 1999), evaluation of this link from "farm to fork" would also seem prudent for *Campylobacter.* An additional concern is that the current consumer

interest in organic and alternative food sources has resulted in some consumers by-passing such food safety measures as pasteurization (Potter, Kaufmann et al. 1984). The practice of drinking raw milk has lead to recent human infections with both *Campylobacter* and Salmonella (Villar, Macek et al. 1999; CDC 2002; CDC 2003). Therefore, unprocessed dairy products may be capable of transmitting not only foodborne pathogens, but also antimicrobial resistance determinants through the exchange of mobile genetic elements such as plasmids or integrons.

This is one of the few studies evaluating the susceptibility of *Campylobacter* by farm type in the United States. In addressing the primary aim of this study to describe the patterns of antimicrobial resistance on organic and conventional dairy farms, our research has demonstrated that *Campylobacter* from dairy farms in the United States is generally susceptible to most antimicrobials. The predominance of *C. jejuni* in cattle isolates has been noted by other authors who employed selective techniques to survey thermophilic *Campylobacter* (Wesley, Wells et al. 2000; Stanley and Jones 2003). Overall, our research agrees with authors who have studied farming systems with more regulated drug use such as the Scandinavian countries (Aarestrup, Nielsen et al. 1997). Aarestrup et al., in 2000 found ampicillin resistance in cattle isolates to be 3%. However, enrofloxacin and nalidixic acid resistance in cattle isolates from the same study was higher (3 % and 14%, respectively) than we reported in our dairy isolates. Since the Danish surveillance program included cattle from slaughter (Bager, Aarestrup et al. 1999), enrofloxacin may have been used in the treatment of beef cattle from which these slaughter samples were taken (Emborg

and Heuer 2002). This drug was approved for veterinary use in 1993 in Denmark (Aarestrup, Jensen et al. 2000). In the United States, fluoroquinolone use in dairy cattle is strictly prohibited. More recent survey data from Denmark collected DANMAP 2001 demonstrated higher levels of erythromycin resistance (8%), streptomycin (13%) (DANMAP 2001). Overall, lower resistance to tetracycline (8%) was found *Campylobacter* isolated from cattle in Denmark (DANMAP 2001) (Aarestrup, Nielsen et al. 1997) than was found in our dairy isolates.

There were two drugs, kanamycin and tetracycline, for which resistance was common to both farm types. The proportion of *Campylobacter* isolates resistant to kanamycin was similar in both organic and conventional farm types ($p = 0.56$). However, level of resistance to tetracycline was significantly higher on conventional farms ($p < 0.007$)). Avrain et al., in 2003 had found associations with tetracycline resistance in broiler chickens to not only be associated to flocks treated with this drug, but also with birds that had been exposed to a coccidiostat only (Avrain, Humbert et al. 2003). Coccidostats are frequently used in conventional dairy heifer rations, but this was not a common practice in our organic herds (Geiger, Ruegg et al. 2003). While studying E. coli isolates, Blake et al, in 2003 found that tetracycline resistance was associated with swine herds under conventional management and was less common among isolates from a dairy animal that was managed organically (Blake, Humphry et al. 2003). However animal exposure or herd use was not ascertained in the study design. Piddock and colleagues evaluated *Campylobacter* susceptibility to five

antimicrobials on dairy farms in the United Kingdom (Piddock, Ricci et al. 2000). The study by Piddock et alk in 2000 was one of few which ascertain both farm use and some individual animal treatment with classes of antimicrobial drugs. Interestingly the work by Piddock and colleagues also found no clear associations between on-farm antimicrobial use and susceptibility patterns in *Campylobacter* isolates to tetracycline, kanamycin, ciprofloxacin, erythromycin, or nalidixic acid (Piddock, Ricci et al. 2000).

Both kanamycin and tetracycline resistance have been described to be carried on plasmids in *Campylobacter* (Taylor, DeGrandis et al. 1981) (Tenover, Fennell et al. 1992). It may be that these mobile genetic elements are continually exchanged between other bacteria and *Campylobacter* despite a lack of selective pressure in the animal host from which it was isolated. Indeed genetic markers for tetracycline resistance have been documented in farming environments (Aminov, Garrigues-JeanJean et al. 2001). Similarly resistance of *Campylobacter* in free living wild birds has also been documented, suggesting that wild life may play a role in the ecology of antimicrobial resistance (Stanley and Jones 1998)

The similarities which we reported here in resistance patterns of the beta-lactam and cephalosporin antibiotics across farm type were surprising. These two drugs are used commonly on conventional dairy farms (Hady, Lloyd et al. 1993) (Geiger, Ruegg et al. 2003). Few of our organic farms reported using these drugs in either their adult cows or in the management of their calves (Geiger, Ruegg et al. 2003). Based on marked difference in usage and current

hypotheses by other researchers in this area (Aarestrup, Seyfarth et al. 2001; Boerlin, Wissing et al. 2001; Evans and Wegener 2003), it is interesting to note that we did not find significant differences between the resistance to beta-lactam or cephalosporin resistance in our *Campylobacter* isolates from conventional or organic farms. This finding warrants further study between actual exposure among conventional on-farm use and also individual animal treatment information.

Some authors have found increases in antimicrobial susceptibility among organic farming systems compared to isolates from conventional farms (Mathew, Beckmann et al. 2001) (Blake, Humphry et al. 2003). The removal of growth promoting antimicrobials has improved the susceptibility profiles of some enteric indicator bacteria (Aarestrup, Seyfarth et al. 2001; Boerlin, Wissing et al. 2001). However, if farms are not prepared for such management changes, animal health can be adversely affected. Caswell et al., in 2003 found declines in animal health, increases in therapeutic antimicrobial use, and also increases in inconsistency of carcass quality following the European ban on growth promoting antimicrobials (Casewell, Friis et al. 2003). Such inconsistency in carcass quality can lead to increased enteric bacterial contamination of meat during the slaughter process and actually increase the risk of foodborne pathogens to humans (Russell 2003).

In other studies, the on-farm use of antimicrobials in the conventional farming system is often assumed in these studies and not actually ascertained

(Blake, Humphry et al. 2003) (Regula, Stephan et al. 2003). Therefore, the conclusion that drug use causes or selects for resistant bacteria must be interpreted with caution when critically evaluating research on this subject. Also, organic farms often tend to be smaller and use very different animal management such as pasture grazing or free range bird environment (Geiger, Ruegg et al. 2003) (Regula, Stephan et al. 2003). These different management practices must be considered in evaluating the ecology of antimicrobial resistance in the farm environment.

Additionally, we have demonstrated that determinants for decreased susceptibility can be found in *Campylobacter* isolated from milk and milk filters, which is particularly worrisome considering that some consumers are bypassing food safety procedures such as pasteurization by purchasing raw milk (Potter, Kaufmann et al. 1984). This behavior has lead to a recent outbreak of milk-borne campylobacterosis in the United States (CDC 2002).

In summary, our findings agree with other authors investigating antimicrobial susceptibility in other bacteria have found little change in cattle isolates over time (Dargatz, Fedorka-Cray et al. 2003) (van Duijkeren, Wannat et al. 2003). In some cases, increasing susceptibility to antimicrobial agents used on dairies has also been documented (Makovec and Ruegg 2003). From our data it also appears that organic farm status does not necessarily translate into a remarkably more susceptible population of *Campylobacter* isolates across all drug classes studied. Both our study findings and trends observed globally demonstrate that the issue of antimicrobial resistance in food animals warrants

continued investigation of herd and individual animal risk factors in order to identify reasonable interventions that insure both food safety and a healthy livestock population.

CHAPTER THREE

Animal-level factors associated with reduced antimicrobial susceptibility of *Campylobacter* isolates from conventional and organic dairy farms

Abstract

As part of a longitudinal study design, the objective of this study was to evaluate animal descriptive parameters as possible risk factors for decreased antimicrobial resistance in Campylobacter. MIC were the outcome for 1122 isolates tested for susceptibility to tetracycline and 854 isolates tested for susceptibility to ampcillin, ciprofloxaxin, ceftriaxone, kanamycin and sulfamethoxazole. Multivariable models were constructed using partial proportional log odds using animal type, health, relative animal age, state, farm type, and animal treats as potential risk factors. Decreased susceptibility to ampicillin was found to be associated with increased odds for calves compared to health cows (OR = 1.5, 95% CI = 1.1-2.0) and associated with lower odds for isolates from organic farm (OR = 0.7, 95% CI = 0.5-0.9) and absence of treatment with a beta lactam (OR=0.2, 95% CI = .1-.5). Decreased susceptibility of kanamycin was associated with increased odds in calves (OR= 4.5, 95% CI 3.3-6.7). Decreased susceptibility in tetracycline was associated with increased odds in calves (OR = 3.7, 95%CI 1.4-6.7). Decreased susceptibility to sulfamethoxazole was associated with decreased odds in the absence of any

treatment (OR=0.4, 95% CI .3-.6) and specifically in absence of treatment with a beta lactam (OR=0.4 , 95% CI .2-.9) or ceftiofur (OR =.4, 95%CI .2 - .8). No animal parameters or herd type were significantly associated with decreasing susceptibility in either ceftriaxone or ciprofloxacin.

Keywords: *Campylobacter*, animal-level risk factors, dairy cattle, organic, dairy farms

1. Introduction

Campylobacter spp. is the most frequently identified cause of bacterial gastroenteritis in the United States (Acheson 2001) (Altekruse and Tollefson 2003). Most *Campylobacter* enteritis cases are mild, self limiting episodes of vomiting, cramping, and diarrhea (Tauxe, Hargrett-Bean et al. 1988) (Altekruse, Swerdlow et al. 1998). However, serious infections with *Campylobacter* may require antimicrobial treatment in infants, geriatric patients, and immune compromised individuals. In these cases bloody stools, dehydration, septicemia, and long-term sequela can occur (Blaser 1997). The demyelinating neurologic disorder Guillian-Barre syndrome (GBS) or intermittent arthritis may follow infections with *Campylobacter* gastroenteritis. (Rees, Soudain et al. 1995) (Nachamkin, Allos et al. 1998). The former occurs subsequent to about 1 in 1000 cases of *Campylobacter* enteritis. Guillian-Barre syndrome is usually transient, but some GBS sufferers continue to have neurologic deficits requiring supportive assistance or care throughout life (Rees, Soudain et al. 1995).

The feces from normally appearing animals may contain *Campylobacter* organisms, since thermophilic *Campylobacter* can colonize the gastrointestinal tracts of mammals and birds without causing disease (Manser and Dalziel 1985). Consequently, human infections have been associated to direct or indirect animal exposure (Deming, Tauxe et al. 1987). While the role that poultry may play in human infections are well-researched (Jacob-Reitsma, Koenraad et al. 1994) (Smith, Besser et al. 1999; Nackamkin, Ung et al. 2002), many human cases and

outbreaks have been linked to cattle sources. Outbreaks of *Campylobacter* enteritis have been associated with raw milk consumption (Warner, Bryner et al. 1986) (Dilworth, Lior et al. 1988) (Kalman, Szollosi et al. 2000) (Lehner, Schneck et al. 2000), dairy farm visits (Evans, Roberts et al. 1996), and water contamination (Duke, Breathnach et al. 1996) (Melby, Svendby et al. 2000) (Frost, Gillespie et al. 2002) It is also known that cattle may be carriers of *Campylobacter* and various levels of prevalence have been documented in dairy animals throughout previous studies. (Green, Kaneene et al. 2001) (Wesley, Wells et al. 2000) (Nielsen 2002).

Campylobacter isolates from humans are displaying increased resistance to many classes of the drugs over time and decreased susceptibility to different drug classes. (Neu 1992) (Engberg, Aarestrup et al. 2000). There is ongoing debate regarding the contribution of human medical, veterinary therapeutic, and animal husbandry practices to the decreased susceptibility of key bacteria to antimicrobials (VanDenBogaard 1997; Smith, Bender et al. 2000; Threlfall, Ward et al. 2000; Wagner, Jabbusch et al. 2003). Also, increased fluoroquinolone resistance has been observed in *Campylobacter* and other bacteria once these antimicrobials were approved in some food animal species(Smith, Besser et al. 1999; McDermott, Bodeis et al. 2002). Also, there has been evidence of increased susceptibility in bacteria when certain antimicrobials were banned from use (Aarestrup, Seyfarth et al. 2001) (Boerlin, Wissing et al. 2001). However, most studies supporting the decrease in susceptibility are based on ecological (aggregative) analysis of data (i.e. which drugs are approved for veterinary use in

a particular country) without ascertaining actual exposure to the drugs being studied. Furthermore, the focus of much research on *Campylobacter* resistance has been on drug classes such as fluoroquinolones and macrolides, while the antimicrobials used on dairy farms are limited. (Hady, Lloyd et al. 1993; Sundlof, Kaneene et al. 1995) It would seem prudent to examine the role of dairy animals to the ecology of potential human exposure to *Campylobacter,* but also the potential to transfer determinants of antimicrobial resistance in this foodborne pathogen.

Therefore, the objective of this study was to assess individual animal parameters including animal type, health status, and antimicrobial treatment history as potential risk factors for decreased antimicrobial susceptibility in *Campylobacter* isolates obtained from individual animals on organic and conventional dairy farms.

2. Materials and Methods

2.1 Farm selection

132 dairy farms were selected from four states: Michigan, Minnesota, New York, and Wisconsin. Data are reported on animal samples from 128 farms from which *Campylobacter* isolates were available for antimicrobial testing. Herds were enrolled according to farm type (organic vs. conventional) and by farm size (number of cows, both milking and dry). To be included in the study, a herd had

to meet the following criteria: 1) at least 30 milking cows, 2) at least 90% of cows of Holstein breed, 3) raise their own calves for replacement cattle, and 4) ship milk all year. Organic farms had to be certified as organic by a recognized organic certification agency and may not have used antimicrobials in cattle greater than 1 year of age for at least 3 years. For conventional farms, lists of farms were obtained from the respective State Departments of Agriculture, and herds within approximately 100 miles of the respective universities were randomly selected to receive a mailing describing the research project. Farms were asked to indicate interest in participation by returning a postcard. The final list of farms was obtained by randomly selecting names of respondents that had indicated willingness to participate. In order to evaluate potential herd management practices as risk factors, a predetermined numbers of farms were enrolled within the following size categories (by number of cows, both milking and dry) of 30-49, 50-99, 100-199, 200 & up. Due to limited availability of organic farms, all known organic farms within approximately 150 miles of the respective universities were contacted to determine eligibility based on the selection criteria and their desire to participate.

2.2 Sample collection

Farms were sampled up to five times from August 2000 through October 2001. For 94% of the farms, the first visit was conducted between October 2000 and January 2001. Subsequent visits to each farm were conducted at approximate 2-month intervals following the first visit.

Cattle samples were collected by placing approximately ten grams of fecal material obtained per rectum into Whirl-Pak® bags. A separate glove was used for the collection of each sample. Since this work is part of a multi-university project, the number of samples collected per herd was based on the prevalence of *Salmonella,* rather than *Campylobacter.* The number collected from specific cattle groups was based on herd size and was calculated to provide similar herd level sensitivity to detect the presence of *Salmonella* assuming the same prevalence for all herds (Warnick, Kanistanon et al. 2003). Calculations resulted in target sample sizes for each visit of 30, 40, 50, and 55 total cattle samples for herds with 30-49, 50-99, 100-199, and ≥ 200 cows, respectively. Systematic sampling was used such that samples were representative of all cattle in each of the following groups on a particular farm on the sampling date: heifer calves receiving milk or milk replacer (preweaned calves), cows to be culled within 14 days (to-be-culled cows), cows due to calve within 14 days (pre-fresh cows) or cows within 14 days after calving (fresh cows), cows designated as "sick" by farm personnel (sick cows), and lactating cows not in any other category (presumed healthy cows). No effort was made to collect samples from the same cattle at subsequent herd visits.

2.3 Shipment and isolation

A central laboratory at Michigan State University, National Food Safety Center was used for all four states. After collection, samples were either taken to the

laboratory (Michigan) or shipped via overnight delivery in styrofoam boxes with ice packs (Minnesota, New York, Wisconsin). Samples were usually shipped the same day as collection but in some cases were also kept in a refrigerator for 12-36 hours before shipping.

Environmental swabs and milk filter were enriched in Bolton broth (Oxoid) containing 5% laked horse blood and selective antimicrobial agents (20mg/L cefaperazone, 20 mg/L vancomycin, 20 mg/L trimethoprim, 50 mg/L cycloheximide). The enriched samples were then incubated at 42° C in 5-10% CO_2 for 48 hours. Animal fecal samples and milk samples were suspended in phosphate buffer saline (PBS) solution. The PBS suspended biological samples and enriched samples were streaked on selective *Campylobacter* Blaser plates (BD Diagnostics,) and incubated at 42° C in 5-10% CO_2 for 48 hours. Typical colonies (small pinpoint gray colonies without hemolysis) were selected and streaked on sheep blood agar (SBA) and incubated at 42°C in 5-10% CO_2 for 48 hours. *Campylobacter* identification was performed from isolated colonies by gram staining, oxidase testing, and motility testing. Hippurate hydrolysis was used to speciate *C. jejuni* using ATCC 33560 as a positive control and *C. coli* as a negative control.

2.4 Antimicrobial susceptibility testing

In vitro susceptibility testing was performed using the microbroth dilution method, following guidelines provided by the National Committee on Clinical

Laboratory Standards (NCCLS) (NCCLS 2003). Bacterial isolates from frozen stock were grown on Brucella agar supplemented with 5% defribrinated sheep blood (BASB) for 48 hours at 42°C under microaerophilic conditions. Individual colonies from each plate were subcultured on BASB under similar growth conditions. Bacteria were swabbed from the BASB and suspended in 5 ml H_2O and the turbidity was adjusted to a 0.5 McFarland standard. This suspension was used to make a 1:10 dilution into Haemophilus testing medium (HTM), resulting in a final bacterial inoculum concentration of approximately 8 x 10^5 CFU/ml.

Customized microbroth dilution plates (CMV1USDA) were purchased pre-made from TREK Diagnostic Systems, Inc., with a prepared range of drug concentrations of azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, and tetracycline (Table 1). *C. jejuni* ATCC33560 and 81176 were used as quality control strains. Each plate was inoculated by adding 100 ul of the bacterial suspension using a Sensititre autoinoculator, covered with a gas-permeable seal, and incubated at 42°C in microaerophilic conditions for 48 hours. The minimum inhibitory concentration (MIC) was determined as the minimum antimicrobial dilution at which no bacterial growth occurred. Following the observation that dairy isolates did not demonstrate resistance patterns similar to humans, another customized antimicrobial panel (CMV2DMSU) was developed with Trek Diagnostics to address drug exposures that are common to dairy cattle management and may allow comparison for animals co-infected with Salmonella. This antimicrobial

panel included 17 drugs encompassing drug classes used on our study farms such as beta lactams & cephalosporins (Geiger, Ruegg et al. 2003).

2.5 Data analysis

Since the outcome of interest is the minimum inhibitory concentration for each antimicrobial, separate log-linear models were developed for ampicillin, kanamycin, sulfamethoxazole, ciprofloxacin, ceftriaxone, and tetracycline.with SAS version 9.0 (Cary, North Carolina). Previous descriptive analysis had demonstrated variability of MIC ranges for ampicillin, kanamycin, sulfamethoxazole and tetracycline for both Campylobacter spp. isolates from organic and conventional dairy farms(Halbert, Kaneene et al. 2003). Cefriaxone and ciprofloxacin were included due to interest human in resistance patterns in foodborne pathogens to these two antimicrobials.(Aarestrup, Jensen et al. 2000; Gupta, Nelson et al. 2004; Kassenborg, Smith et al. 2004)

Proportional odds criteria were evaluated for each log-linear model. In order to apply log-linear models to ordinal outcomes such as MICs, the data must fulfill the proportional odds assumption(Stiger, Barnhart et al. 1999). For the data presented here, it was determined that there was significant violation of the assumption of identical log-odds each antimicrobial in this study. If proportional odds were used in violation of the above assumption, the model would be likely to result in misspecification of the estimates based on parallel slopes regardless of where the data dichotomization was assigned (Ananth and Kleinbaum 1997) (Stokes, Davis et al. 2003). Therefore, in order to assess the distributions of MIC as dependent variables, partial proportional odds were used using

generalized estimating equation (GEE) methodology. In order to accomplish this, dummy variables consisting of logits were created for each observation by the MICs for each antimicrobial. The use of partial proportional odds allows variability in the log odds across possible dichotomization of MIC comparison levels (Ananth and Kleinbaum 1997). Each isolate was considered an observation and the REPEATED statement was used in Proc GenMod for each isolate with the respective logits of MIC as dependent outcomes using an exchangeable working correlation structure account for the isolate serving as a random effect.(Stokes, Davis et al. 2003) using SAS version 9.0 (Cary, North Carolina).

For all models, a backward stepwise process was used to fit the final model by initially evaluating a fully parameterized model of all risk factors with $p < 0.20$ (Agresti 1999). Variables were removed in a stepwise manner by those with the highest p-value first based Type 3 GEE Analysis (F-test) until all variables left in the model had $p < 0.05$ and overall goodness of fit (Ananth and Kleinbaum 1997) . Animal-level variables evaluated included animal type (healthy cow, pre-fresh cow, fresh cow, cull cow, sick cow, or pre-weaned calf), animal age (using lactation number as a proxy), animal health status, treatment with the antimicrobial class for the outcome of interest or related drug class (such as beta-lactams and cephalosporins), and other antimicrobial treatments with other drugs. Although not descriptive parameters of individual animals, herd enrollment criteria, including farm type (organic or conventional) and state of enrollment were evaluated as possible confounding variables.

3. Results

3.1 Distribution of Isolates and Individual Animal Risk Factors

Over 97% of our isolates were classified as *C. jejuni* (Green, Kaneene et al. 2001). The summary of the descriptive statistics for individual-animal level risk factors used to develop each antimicrobial model are presented in Table 8 and Table 9. Since tetracycline was included in the original 8-drug microbroth dilution panel, the 1122 *Campylobacter spp* isolates tested with this panel and their associated animal-level descriptive parameters are included in Table 8. Descriptive statistics for the 854 *Campylobacter spp* isolates that were tested with the 17-drug antimicrobial panel for ampicillin, ceftriaxone, ciprofloxacin, sulfamethoxazole and kanamycin subjected to microbroth dilution for are included in Table 9 In selecting isolates for antimicrobial testing for either 8-drug or 17-drug panel, distributing isolates by farm type, animal classification, state of origin and known treatments was emphasized as displayed (Table 8 and Table 9). Due to the limitations of antimicrobials used in dairies, isolates from cattle with treatments with a fluoroquinolone, tetracycline, sulfa-drug, or macrolide were uncommon among our Campylobacter spp isolates (Tables 8and 9. However treatments with a ceftiofur or Beta-lactam (penicillin or ampicillin) were relatively common at 3.9% and 3.5% of isolates, respectively (Table 9).

Table 8: Descriptive Statistics for Tetracycline Susceptibility Isolates (n=1122)

	Variable	Levels	Number of Isolates	Proportion of Isolates
Explanatory Variables	Farm Type	1- Organic	282	25.1%
		2- Conventional	840	74.9%
	State	1- Michigan	286	25.5%
		2- Minnesota	312	27.8%
		3- New York	274	24.4%
		4- Wiscosin	250	22.3%
	Animal Type	1 – Healthy Cows	532	47.4%
		2 – Pre Fresh Cow	44	3.9%
		3 – Fresh Cow	84	7.5%
		4 – Cull Cow	16	1.4%
		5 – Sick Cow	60	5.4%
		6 – Pre Weaned Calf	386	34.4%
	Parity	0- Calf/Heifer	394	35.2%
		1 – 1st Lactation	305	27.3%
		2 – 2nd Lactation	208	18.6%
		3 – 3rd Lactation	100	8.9%
		4 – 4th Lactation	56	5.0%
		5 – 5th Lact and +	50	4.9%
	Health Status	0 - Healthy	1050	93.8%
		1 – Metritis	16	1.4%
		2 – Mastitis	8	0.7%
		3 – Pneumonia	5	0.5%
		4 – Ketosis	3	0.3%
		5 – LDA or RDA	10	0.9%
		6 – Lame	9	0.8%
		7 – Diarrhea/scours	14	1.3%
		8 – Milk Fever	1	0.1%
		9 – Peritonitis	1	0.1%
		10- Hardware	2	0.2%
	Treated With Tetracycline	1-Yes	3	0.3%
		0 - No	1119	99.7%
	Treated with other Antimcirobial	1 – Yes	35	3.1%
		0 – No	1097	96.9%
Dependent Variable	Tetracycline MIC (ug/ml)	0.25	430	38.3%
		0.5	18	1.6%
		1	3	0.3%
		2	0	0%
		4	5	0.5%
		8	19	1.7%
		16	74	6.6%
		32	166	14.8%
		64	260	23.2%
		128	117	10.4%
		256	30	2.7%

Table 9: Descriptive Statistics for Ampicillin, Ciprofloxacin, Ceftriaxone, Kanamycin, and Sulfamethoxazole Susceptibility Isolates (n=854)

	Variable	Levels	Number of Isolates	Proportion of Isolates
Explanatory Variables	Farm Type	1- Organic	168	19.7%
		2- Conventional	686	80.3%
	State	1- Michigan	229	26.8%
		2- Minnesota	233	27.3%
		3- New York	201	23.5%
		4- Wiscosin	191	22.4%
	Animal Type	1 – Healthy Cows	389	45.5%
		2 – Pre Fresh Cow	59	6.9%
		3 – Fresh Cow	128	15.0%
		4 – Cull Cow	19	2.2%
		5 – Sick Cow	86	10.1%
		6 – Pre Weaned Calf	173	20.3%
	Parity	0- Calf/Heifer	186	21.7%
		1 – 1st Lactation	273	32.0%
		2 – 2nd Lactation	205	24.0%
		3 – 3rd Lactation	94	11.0%
		4 – 4th Lactation	54	6.3%
		5 – 5th Lact and +	42	4.9%
	Health Status	0 - Healthy	729	85.4%
		1 – Metritis	24	2.8%
		2 – Mastitis	12	1.4%
		3 – Pneumonia	15	1.8%
		4 – Ketosis	4	0.5%
		5 – LDA or RDA	27	3.2%
		6 – Lame	16	1.9%
		7 – Diarrhea/scours	26	3.0%
		8 – Milk Fever	0	0%
		9 – Peritonitis	0	0%
		10- Hardware	1	0.1%
	Rx with Pen/Amp	0-Not Rx	824	96.5%
		1-Rx with	30	3.5%
	Rx with Ceftiofur	0-Not Rx	821	96.1%
		1- Rx with	33	3.9%
	Rx with Macrolide	0-Not Rx	850	99.5%
		1-Rx with	4	0.5%
	Rx with fluoroquinolone	0-Not Rx	851	99.6%
		1-Rx with	3	0.4%
	Rx with Sulfa	0-Not Rx	849	99.4%
		1-Rx with	5	0.6%
		4	279	32.7%
		8	269	31.5%
		16	69	8.1%
		32	13	1.5%
		64	58	6.8%

Table 9 (cont'd)

Dependent Variables	Ciprofloxacin MIC (ug/ml)	0.5	844	98.8%
		1	2	0.2%
		2	2	0.2%
		4	1	0.1%
		8	4	0.5%
		16	1	0.1%
	Sulfa MIC (ug/ml)	64	45	5.3%
		128	185	21.67%
		256	304	35.6%
		512	320	37.5%
	Kanamycin MIC (ug/ml)	8	527	61.7%
		16	53	6.2%
		32	2	0.2%
		64	1	0.1%
		128	271	31.7%
	Ceftriaxone MIC (ug/ml)	4	55	6.4%
		8	244	28.6%
		16	352	41.2%
		32	189	22.1%
		64	14	1.6%
	Ampicillin MIC (ug/ml)	2	166	19.4%
		4	279	32.7%
		8	269	31.5%
		16	69	8.1%
		32	13	1.5%
		64	58	6.8%

3.2 Ampicillin

Animal type, state of origin, treatment with a beta-lactam and herd type were found to be significant in the final multi-variable model (Table 10). Calves had an increased odds of reduced susceptibility compared to healthy mature cows in the herd. There was a tendency for cull cows, sick cows and pre-fresh cows to have reduced susceptibility (Table 10). However, this trend was not statistically significant ($p > 0.05$). *Campylobacter* isolates from Wisconsin and Michigan displayed reduced susceptibility compared to isolates from New York. Isolates from Minnesota were not significantly different susceptibility (Table 10). Animals that had not received a treatment were significantly lower odds of

reduced susceptibility to ampicillin compared to cattle that had been treated with a beta-lactam. Isolates from organic farms had significantly lower odds for decreased susceptibility compared to *Campylobacter* isolates from conventional farms (Table 10). Treatment with other antimicrobials including ceftiofur, animal health status, and relative animal were not associated with decreased susceptibility to ampicillin in our *Campylobacter* isolates.

Table 10: Ampicillin Decreased Susceptibility - Final Multivariable Model

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Cattle type	6 – Pre Weaned Calf	1.5	1.1	2.0
	5 – Sick Cow	1.1	0.7	1.7
	4 – Cull Cow	0.4	0.1	1.1
	3 – Fresh Cow	1.2	0.8	1.8
	2 – Pre Fresh Cow	1.1	0.7	1.8
	1 – Healthy Cows			
State	WI	1.5	1.1	2.2
	MI	1.3	1.1	2.0
	MN	1.1	0.7	1.8
	NY	1.0		
Beta-Lactam	UnTreated	0.2	0.1	0.5
	Treated	1.0		
Farm Type	Organic	0.7	0.5	0.9
	Conventional	1.0		

3.3 Kanamycin

Animal type and state of origin were retained in the final model for reduced susceptibility to kanamycin in our *Campylobacter* isolates (Table 11). Isolates from pre-weaned calves were at 4.5 times the odds of reduced susceptibility compared to isolates from healthy mature cows. Isolates from cull cows demonstrated a tendency toward reduced susceptibility and isolates from fresh cows had lower odds of reduced susceptibility (Table 11). However, the findings of isolate susceptibility in cull and fresh cows were not significantly different from isolates from healthy cows. *Campylobacter* isolates from Michigan were at significantly reduced odds to demonstrate reduced susceptibility compared to isolates from Wisconsin. Isolates from Minnesota and New York were similar in susceptibility to kanamycin (Table 11). Treatment with a macrolide, treatment with any antimicrobial, relative animal age, farm type and animal health status were not associated with decreased susceptibility to kanamycin in our *Campylobacter* isolates.

Table 11: Kanamycin Decreased Susceptibility - Final Multivariable Model

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Cattle type	6 – Pre Weaned Calf	4.5	3.3	6.7
	5 – Sick Cow	0.9	0.6	1.6
	4 – Cull Cow	1.6	0.6	4.0
	3 – Fresh Cow	0.8	0.5	1.3
	2 – Pre Fresh Cow	0.9	0.5	1.7
	1 – Healthy Cows			
State	MI	0.6	0.4	0.9
	MN	1.1	0.7	1.6
	NY	0.9	0.6	1.3
	WI	1.0		

3.4 Tetracycline

Animal type and state of origin were significantly associated with reduced susceptibility to tetracycline (Table 12). Isolates from pre-weaned calves were at 3.1 times the odds of reduced susceptibility compared to isolates from pre-fresh cows. Isolates from cull cows demonstrated increased odds toward reduced susceptibility and isolates from fresh cows, healthy cows, and sick cows had lower odds of reduced susceptibility (Table 12). However, these findings were not significantly different from isolates from pre-fresh cows. Campylobacter isolates from Wisconsin and New York were at significantly reduced odds to demonstrate reduced susceptibility compared to isolates from Minnesota. Isolates from Michigan did not differ in odds of susceptibility to isolates from Minnesota (Table 12). Farm type, animal health status, relative animal age,

treatment with tetracycline, and treatment with any antibiotic were not associated with decreased susceptibility to tetracycline.

Table 12: Tetracycline Decreased Susceptibility - Final Multivariable Model

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Cattle type	6 – Pre Weaned Calf	3.1	1.4	6.7
	5 – Sick Cow	0.5	0.2	1.5
	4 – Cull Cow	1.4	0.3	5.9
	3 – Fresh Cow	0.9	0.4	2.4
	1– Healthy Cows	0.7	0.3	1.6
	2 – Pre Fresh Cow			
State	WI	0.5	0.3	0.8
	MI	0.9	0.6	1.3
	NY	0.6	0.4	0.9
	MN	1.0		

3.5 Sulfamethoxazole

Animal treatment history was significantly associated with odds of decreased susceptibility to sulfamethoxazole. Animals that had not been treated at all or specifically had not received either a beta-lactam or ceftiofur had reduced odds of decreased susceptibility to sulfamethoxazole (Table 13). Therefore, treatment with a beta-lactam or treatment with ceftiofur was associated with increased odds of decreased susceptibility. Treatment with a sulfa drug, animal health status, relative animal age, state, and farm type were

not associated with decreased susceptibility to sulfamethoxazole in our *Campylobacter* isolates.

Table 13: Sulfamethoxazole Decreased Susceptibility - Final Multivariable Model

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Other Rx	UnTreated	0.4	0.3	0.6
	Treated with a drug besides Sulfa	1		
Beta-Lactam	Un Treated	0.4	0.2	0.9
	Treated	1		
Ceftiofur	Un Treated	0.4	0.2	0.8
	Treated	1		

3.6 Ceftriaxone

Two parameters, (ceftiofur treatment and farm type) demonstrated p-values on univariable analysis for inclusion in a multivariable model, p=0.07 and p=.08 respectively. However, both variables could not support a multivariable model and retain a significant p value. Therefore, none of the individual animal risk factors or enrollment parameters was found to be associated with decreased susceptibility of ceftriaxone, including treatment with ceftiofur, treatment with a beta-lactam, treatment with any antimicrobial, animal health status, relative animal age, farm type, or state of enrollment.

3.7 Ciprofloxacin

No variables were significant on univariable analysis in order to be able to build a multivariable model. Therefore, farm type, state of enrollment, animal

health status, relative animal age, treatment with a fluorquionolone, and treatment with any antimicrobial were not significantly associated with decreasing susceptibility of our *Campylobacter* isolates. Even treatment with a fluoroquinolone only resulted in a p=0.39.

4. Discussion

Particular strengths of this study included the number of herds sampled in four different states in the United States, diversity of farms sampled (both in management style and herd sizes), actual individual animal treatment records over time, and sampling from individual animal types within each farm. As a longitudinal study conducted over the course of one year, animal types could be sampled from a diversity of farms and treatments recorded as they occurred (Geiger, Ruegg et al. 2003). The development of an expanded antimicrobial panel allowed the assessment of susceptibilities for drugs used on dairy farms, which are not captured when only antimicrobials of interest to treat human infections are included in the study design.

The use of log-linear models allows the evaluation of an ordinal outcome variable such as a minimum inhibitory concentration, rather that simply dichotomizing the data. Currently, there is little standardization in the global microbiological community regarding the interpretive criteria for the antimicrobial susceptibility of *Campylobacter*. Breakpoints for the classification of *Campylobacter* as resistant are often those used for other enteric pathogens, which may not be clinically relevant to *Campylobacter*. Similarly, the use of MIC_{50} and MIC_{90} has been used

by some authors (Aarestrup, Nielsen et al. 1997) . However, we found that MIC_{50} and MIC_{90} are not sensitive in detecting differences between the *Campylobacter* antimicrobial susceptibility from organic and conventional dairy farms (Halbert, Kaneene et al. 2003).Therefore, by using generalized estimating equations (GEE) with partial proportional odds, this study was able to assess more subtle differences in susceptibility by treating MIC as an ordinal outcome across for each antimicrobial by the individual animal risk factors for each *Campylobacter* isolate (Stiger, Barnhart et al. 1999).

Of the primary exposure variables of antibiotic treatments and farm type (conventional compared to organic dairies), there was inconsistency in our findings. Only decreased susceptibility to ampicillin was associated to both conventional farm type and treatment with a beta-lactam. Farm type and treatment with the antimicrobial of interest were not associated with increased odds of decreased susceptibility in the other antimicrobial models. This supports a recent study of Campylobacter in which the proportion of resistant isolates did not differ significantly between organic or conventional dairy farm for ciprofloxacin, gentamicin, erythromycin or tetracycline (Sato, Bartlett et al. 2004). Interestingly the use of a third generation cephalosporin, ceftiofur, was not associated to co-selection of decreased susceptibility to ampicillin. Ceftiofur is one of the most commonly used antimicobials in dairies including those enrolled in this study. (Geiger, Ruegg et al. 2003) We also did not find an association with ceftiofur use in individual animals and decreased susceptibility to ceftriaxone as has been asserted by other authors in foodborne pathogens, such as

Salmonella (Fey, Safranek et al. 2000). However, these Fey et al., did not document use of this antibiotic on the cattle farms where ceftriaxone-resistant Salmonella was isolated. Therefore, the role of cattle exposure to ceftiofur in their findings (Fey, Safranek et al. 2000) is unknown.

One of the more consistent findings in the antimicrobial susceptibilities described in this study is the association of animal type. Calves were at higher odds of decreased susceptibility in ampicillin, kanamycin, and tetracycline. Only in the case of ampicillin, was the association of animal type also in conjunction with an antibiotic treatment with the drug class of interest. The differential antimicrobial susceptibility in young animals has been documented by other authors in other enteric bacteria such as *E. coli* (Berge, Atwill et al. 2003) (Orden, Ruiz-Santa et al. 2000). The observation of higher tetracycline resistance in calves compared to cows was also recently documented in dairy farms (Sato, Bartlett et al. 2004). It is quite plausible that the pre-ruminating calf represents a very different ecology for *Campylobacter* and may select for other survival or fitness traits such as efflux pumps and other molecular determinants which might also be reflected increased susceptibility (Pumbwe, Randall et al. 2004) (Berge, Atwill et al. 2003). The *pTet* plasmid in Campylobacter jejuni has been recently sequenced, which determined that sequences consistent with type IV secretions systems are present (Batchelor, Pearson et al. 2003). Both kanamycin and tetracycline resistance have been determined to be carried on a plasmid (Kotarski, Merriwether et al. 1986; Taylor 1986). Therefore, *Campylobacter* strains that maintain a plasmid with probably secretion systems such as *pTet* with many

imilar sequences to the *pVir* plasmid may serve an advantage in colonizing reruminants or non-ruminating animals (Bacon, Alm et al. 2000; Bacon, Alm et l. 2002; Batchelor, Pearson et al. 2003). Another possible exposure to these nobile genetic elements may be through the environment where calves are noused, since genetic markers for tetracycline resistance have been documented in farming environments (Aminov, Garrigues-JeanJean et al. 2001).

The location of the dairy farm by state of enrollment was also significantly associated with odds of decreased susceptibility in several of the antimicrobial models. However, the direction of the association was not consistent in all models. Wisconsin and New York *Campylobacter* isolates had decreased odds for decreased tetracycline susceptibility. However, Wisconsin and Michigan had increased odds of decreased susceptibility to ampicillin and isolates from Michigan had decreased odds of decreased susceptibility to kanamycin. It is possible that these findings reflect differing herd management within the states or perhaps patterns in veterinary treatment which may reflect differences in veterinary education at the respective veterinary colleges in each of the four states. Often management on dairy farms is passed on from generation to generation and varies by geography. Also, new veterinary graduates may develop their practice habits from senior partners in the veterinary practices they join. However, these associations are beyond the scope of this individual-animal level investigation.

Some studies have found significant resistance to fluorquinolones in cattle isolates (Aarestrup, Bager et al. 1998). However, our work could not demonstrate any significant associations between animal risk factors or farm type with decreased susceptibility to ciprofloxacin. In this study, we had few *Campylobacter* isolates recovered from animals treated with a fluoroquinolone. It is unknown if treatment with a fluoroquinolone may be associated with reduced likelihood of recovering *Campylobacter* from cattle. Overall, use of fluoroquionolines in dairy animals is uncommon and use of this drug class was documented to be rare in this longitudinal study both on a herd and individual animal-level (Geiger, Ruegg et al. 2003).

5. Conclusion

In summary, our findings agree with other authors investigating antimicrobial susceptibility in which no clear associations were identified between on-farm antimicrobial use and susceptibility patterns in *Campylobacter* isolates to tetracycline, kanamycin, ciprofloxacin, erythromycin, or nalidixic acid (Piddock, Ricci et al. 2000). In some cases, increasing susceptibility to antimicrobial agents used on dairies has also been documented (Makovec and Ruegg 2003). From our data, it would appear that further study on the ecology of *Campylobacter* on dairy farms and management practices may shed light on the observations of this study. While many authors on the subject of antimicrobial resistance are ready to make a causal association between use of antimicrobial agents in food animals and antibiotic resistance in foodborne pathogens, our work does not indicate that

treatment of dairy cattle with antimicrobials results in clearly decreased susceptibility in *Campylobacter* isolates.

CHAPTER FOUR

Herd-level management factors associated with reduced antimicrobial susceptibility of *Campylobacter* isolates from conventional and organic dairy farms

Abstract

Using a longitudinal study design, herd management practices were assessed as risk factors for decreased antimicrobial resistance in *Campylobacter* isolated from 97 conventional and 31 organic dairy farms. Minimum inhibitory concentrations were determined for ceftriaxone, sulfamethoxazole, ampicillin, kanamycin, tetracycline and cirpfloxacin. MIC50 were calculated for each herd and used as the outcome of interest to develop multivariable proportional log-odd models for the herd management parameters for both herd types. For ceftriaxone susceptibility on conventional farms, lack of using a disinfect to clean milk buckets was associated with increased odds (OR =3.3). If the farm did not use sulfa drugs a decreased odds was found (OR=0.1). For organic farm, cetriaxone decreased susceptibility was associated to increased odds if no coccidiostat was used (OR=10.7). However, the use of a designated sick pen was associated with decreased odds (OR =0.1). Sulfamethoxazole decreased susceptibility on conventional dairy farms was associated with decreased odds for farm with low SCC (OR =.02) and no access to surface water (OR= 0.3) and lack of florfenicol on the farm (OR=0.1). Sulfamethoxazole decreased susceptibility was associated to a reduced odds if cattle did not have access to

surface water (OR=0.3). Kanamycin decreased susceptibility in conventional farms was associated to higher odds if no core antigen vaccine was used (OR=3.1) and less than 3 quarts of colostrem were fed (OR= 5.8). Lower odds were associated to farms that did not graze (OR= 0.3) or use a transition ration (0.3). The most significant factor of decreased susceptibility for kanamycin on organic farm was group housing of calves, either in pens (OR = 78) or in calf barns (OR= 37), compared to calves hutches. Reduced susceptibility on both farm types was associated to increased odds if hutches were not moved in between calves.

Keywords: *Campylobacter*, herd-level risk factors, dairy cattle, organic, dairy farms

1. Introduction

Campylobacter spp. is one of the most frequently identified causes of bacterial gastroenteritis in the United States and many areas around the world (Petersen, Nielsen et al. 2001; Burch 2002; Padungtod and Kaneene 2003) (Altekruse and Tollefson 2003). While most cases of *Campylobacter* enteritis cases are mild, self limiting episodes of vomiting, cramping, and diarrhea (Tauxe, Hargrett-Bean et al. 1988) (Altekruse, Swerdlow et al. 1998), patients such infants, geriatric patients, and immune compromised individuals may require antimicrobial therapy. In these cases, efficacy of the antimicrobial chosen to treat the infection is crucial. However, a concerning trend is that *Campylobacter* isolates from humans are displaying increased resistance to many classes of antimicrobials (Neu 1992) (Engberg, Aarestrup et al. 2000).

There is also ongoing debate regarding the contribution of human medical, veterinary therapeutic, and animal husbandry practices to the decreased susceptibility of key bacteria to antimicrobials (VanDenBogaard 1997; Smith, Bender et al. 2000; Threlfall, Ward et al. 2000; Wagner, Jabbusch et al. 2003). However, most studies supporting the decrease in susceptibility are based on ecological (aggregative) analysis of data (i.e. which drugs are approved for veterinary use in a particular country) without ascertaining actual exposure to the drugs being studied. However, some time order is presumed, since increased fluoroquinolone resistance in *Campylobacter* and other bacteria has been noted once these antimicrobials were approved in some food animal species(Smith, Besser et al. 1999; McDermott, Bodeis et al. 2002). There has been evidence of

increased susceptibility in bacteria when certain antimicrobials were banned from use (Aarestrup, Seyfarth et al. 2001) (Boerlin, Wissing et al. 2001). However, none of these studies controlled for farm management practices which may impact the ecology of enteric bacteria within a farm population.

Healthy adult cows and calves may be colonized by *Campylobacter* and numerous studies have documented varying prevalence levels in dairy animals (Green, Kaneene et al. 2001) (Wesley, Wells et al. 2000) (Nielsen 2002). More importantly, a number of human outbreaks of *Campylobacter* enteritis have been associated with raw milk consumption (Warner, Bryner et al. 1986) (Dilworth, Lior et al. 1988) (Kalman, Szollosi et al. 2000) (Lehner, Schneck et al. 2000), dairy farm visits (Evans, Roberts et al. 1996), and water contamination (Duke, Breathnach et al. 1996) (Melby, Svendby et al. 2000) (Frost, Gillespie et al. 2002). However, the focus of antimicrobial resistance in *Campylobacter* has been on drug classes such as fluoroquinolones and macrolides, while study of antimicrobial susceptibility in *Campylobacter* by drug classes used on dairy farms are lacking (Hady, Lloyd et al. 1993; Sundlof, Kaneene et al. 1995). Thus, the role of dairy farm practices to the development of antimicrobial resistance in *Campylobacter* remains poorly defined despite numerous outbreaks of enteritis that have been directly associated with dairy sources.

Therefore, the objective of this study was to assess the association between dairy farm management practices including on-farm use of antibiotics and

decreased antimicrobial susceptibility of *Campylobacter* isolates obtained from individual animals on organic and conventional dairy farms.

2. Materials and Methods

2.1 Farm selection

132 dairy farms were selected from four states: Michigan, Minnesota, New York, and Wisconsin. Data are reported on animal samples from 128 farms from which *Campylobacter* isolates were available for antimicrobial testing. Herds were enrolled according to farm type (organic vs. conventional) and by farm size (number of cows, both milking and dry). To be included in the study, a herd had to meet the following criteria: 1) at least 30 milking cows, 2) at least 90% of cows of Holstein breed, 3) raise their own calves for replacement cattle, and 4) ship milk all year. Organic farms had to be certified as organic by a recognized organic certification agency and may not have used antimicrobials in cattle greater than 1 year of age for at least 3 years. For conventional farms, lists of farms were obtained from the respective State Departments of Agriculture, and herds within approximately 100 miles of the respective universities were randomly selected to receive a mailing describing the research project. Farms were asked to indicate interest in participation by returning a postcard. The final list of farms was obtained by randomly selecting names of respondents that had indicated willingness to participate. In order to evaluate potential herd management practices as risk factors, a predetermined numbers of farms were enrolled within the following size categories (by number of cows, both milking

and dry) of 30-49, 50-99, 100-199, 200 & up. Due to limited availability of organic farms, all known organic farms within approximately 150 miles of the respective universities were contacted to determine eligibility based on the selection criteria and their desire to participate.

2.2 Sample collection

Farms were sampled up to five times from August 2000 through October 2001. For 94% of the farms, the first visit was conducted between October 2000 and January 2001. Subsequent visits to each farm were conducted at approximate 2-month intervals following the first visit.

Cattle samples were collected by placing approximately ten grams of fecal material obtained per rectum into Whirl-Pak® bags. A separate glove was used for the collection of each sample. Since this work is part of a multi-university project, the number of samples collected per herd was based on the prevalence of *Salmonella,* rather than *Campylobacter.* The number collected from specific cattle groups was based on herd size and was calculated to provide similar herd level sensitivity to detect the presence of *Salmonella* assuming the same prevalence for all herds (Warnick, Kanistanon et al. 2003). Calculations resulted in target sample sizes for each visit of 30, 40, 50, and 55 total cattle samples for herds with 30-49, 50-99, 100-199, and ≥ 200 cows, respectively. Systematic sampling was used such that samples were representative of all cattle in each of the following groups on a particular farm on the sampling date: heifer calves receiving milk or milk replacer (preweaned calves), cows to be culled within 14 days (to-be-culled cows), cows due to calve within 14 days (pre-fresh cows) or

cows within 14 days after calving (fresh cows), cows designated as "sick" by farm personnel (sick cows), and lactating cows not in any other category (presumed healthy cows). No effort was made to collect samples from the same cattle at subsequent herd visits.

2.3 Shipment and isolation

A central laboratory at Michigan State University, National Food Safety Center was used for all four states. After collection, samples were either taken to the laboratory (Michigan) or shipped via overnight delivery in styrofoam boxes with ice packs (Minnesota, New York, Wisconsin). Samples were usually shipped the same day as collection but in some cases were also kept in a refrigerator for 12-36 hours before shipping.

Environmental swabs and milk filter were enriched in Bolton broth (Oxoid) containing 5% laked horse blood and selective antimicrobial agents (20mg/L cefaperazone, 20 mg/L vancomycin, 20 mg/L trimethoprim, 50 mg/L cycloheximide). The enriched samples were then incubated at 42° C in 5-10% CO_2 for 48 hours. Animal fecal samples and milk samples were suspended in phosphate buffer saline (PBS) solution. The PBS suspended biological samples and enriched samples were streaked on selective *Campylobacter* Blaser plates (BD Diagnostics,) and incubated at 42° C in 5-10% CO_2 for 48 hours. Typical colonies (small pinpoint gray colonies without hemolysis) were selected and streaked on sheep blood agar (SBA) and incubated at 42°C in 5-10% CO_2 for 48

6.

hours. *Campylobacter* identification was performed from isolated colonies by gram staining, oxidase testing, and motility testing. Hippurate hydrolysis was used to speciate *C. jejuni* using ATCC 33560 as a positive control and *C. coli* as a negative control.

2.4 Antimicrobial susceptibility testing

In vitro susceptibility testing was performed using the microbroth dilution method, following guidelines provided by the National Committee on Clinical Laboratory Standards (NCCLS) (NCCLS 2003). Bacterial isolates from frozen stock were grown on Brucella agar supplemented with 5% defribrinated sheep blood (BASB) for 48 hours at 42°C under microaerophilic conditions. Individual colonies from each plate were subcultured on BASB under similar growth conditions. Bacteria were swabbed from the BASB and suspended in 5 ml H_2O and the turbidity was adjusted to a 0.5 McFarland standard. This suspension was used to make a 1:10 dilution into Haemophilus testing medium (HTM), resulting in a final bacterial inoculum concentration of approximately 8×10^5 CFU/ml.

Customized microbroth dilution plates (CMV1USDA) were purchased pre-made from TREK Diagnostic Systems, Inc., with a prepared range of drug concentrations of azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, and tetracycline (Table 1). *C. jejuni* ATCC33560 and 81176 were used as quality control strains. Each plate was

inoculated by adding 100 ul of the bacterial suspension using a Sensititre autoinoculator, covered with a gas-permeable seal, and incubated at 42°C in microaerophilic conditions for 48 hours. The minimum inhibitory concentration (MIC) was determined as the minimum antimicrobial dilution at which no bacterial growth occurred. Following the observation that dairy isolates did not demonstrate resistance patterns similar to humans, another customized antimicrobial panel (CMV2DMSU) was developed with Trek Diagnostics to address drug exposures that are common to dairy cattle management and may allow comparison for animals co-infected with Salmonella. This antimicrobial panel included 17 drugs encompassing drug classes used on our study farms such as beta lactams & cephalosporins (Geiger, Ruegg et al. 2003).

2.5 Data analysis

The outcome of interest for the herd-level analysis is the minimum inhibitory concentration that inhibits the growth of half of the isolates from each farm (MIC_{50}) for each antimicrobial. Due to the ordinal nature of this outcome, separate log-linear models were developed for ampicillin, kanamycin, sulfamethoxazole, ceftriaxone, and tetracycline. Previous descriptive analysis had demonstrated variability of MIC_{50} ranges for ampicillin, kanamycin, sulfamethoxazole and tetracycline for *Campylobacter* isolates from organic and conventional dairy farms (Halbert, Kaneene et al. 2001). Cefriaxone and ciprofloxacin were evaluated due interest human resistance patterns in foodborne pathogens to these two antimicrobials.(Aarestrup, Jensen et al. 2000; Gupta, Nelson et al. 2004; Kassenborg, Smith et al. 2004). The MIC_{50} for all

herds for ciprofloxacin was 0.5 ug/ml; therefore, risk factors for decreased susceptibility for this antimicrobial could not be modeled.

Variables that were assessed for association to herd susceptibility for each antimicrobial were obtained from data collected through data collection instruments administered at the initial herd visit and subsequent bi-monthly sampling visits. General herd descriptive variables included herd size, herd type, and location by state. Parameters for milk production and milk quality included rolling herd average (categorized by quartiles), somatic cell count and milk raw bacterial count. Cattle housing was included as level of exposure, including multiple loose cattle, individual animal stalls, or hutches. Animal health variables included the quartile scores for reported herd morbidity due to diarrhea and mortality, and categories for proportion of animals treated in calf and cow populations. Variables for other animal exposure included a score for other species present on the farm and level of rodent control. Cats were excluded from this variable since all farms except four reported cats on the premises. Hygiene was characterized by methods of manure and feed handling, cleaning of calf feeding equipment and calf pens, separation of maternity and sick cow housing, access to surface water, and grazing access to land where manure was spread. General feed management descriptive variables included use of a total mixed ration (TMR), transition ration, feeding of anionic salts, and animal sources of protein and fat. The use of coccidastats and medicated milk replacer or waste milk use was also included. Antimicrobial use was characterized by level of *Beta*-lactam use (penicillin, amoxicillin, or ampicillin), level of third-generation

cephalosporin (ceftiofur) use, level of tetracycline use, and dichotomous variables for sulfa drug and florfenicol use. Management of animal treatment was assessed by the use of the herd veterinarian for recommended therapy and herd records maintained for both the calf and cow populations.

Proportional odds criteria were evaluated for each log-linear model. In order to apply log-linear models to ordinal outcomes such as MIC_{50}, the data must fulfill the proportional odds assumption (Stiger, Barnhart et al. 1999). For the data presented here, the distributions of MIC_{50} for the herd outcome of ceftriaxone, sulfamethoxazole, and kanamycin fulfilled the assumption of proportional log odds. However, it was determined that there was significant violation of the assumption of identical log-odds for ampicillin and tetracycline in herd MIC_{50}. If proportional odds were used in violation of the above assumption, the model would be likely to result in misspecification of the estimates based on parallel slopes regardless of where the data dichotomization was assigned (Ananth and Kleinbaum 1997) (Stokes, Davis et al. 2003). Therefore, in order to assess the distributions of MIC_{50}as dependent variables, partial proportional odds were computed using generalized estimating equation (GEE) methodology. In order to accomplish this, dummy variables consisting of logits were created for each observation by the MICs for each antimicrobial. The use of partial proportional odds allows variability in the log odds across possible dichotomization of MIC_{50}comparison levels (Ananth and Kleinbaum 1997). Each isolate was considered an observation and the REPEATED statement was used in Proc GenMod for each StudyID with the respective logits of MIC_{50} as dependent

outcomes using an exchangeable working correlation structure account for the StudyID serving as a random effect.(Stokes, Davis et al. 2003) using SAS version 9.0 (Cary, North Carolina).

For all models, a backward stepwise process was used to fit the final model by initially evaluating a fully parameterized model of all herd management risk factors with $p < 0.20$ based on univariable analysis (Agresti 1999). Variables were removed in a stepwise fashion, removing those with the highest p-value first based on Type 3 GEE Analysis (F-test) until all variables left in the model had $p < 0.05$ using each Proc Logistic or Proc GenMod for proportional log odds (Stokes, Davis et al. 2003) or partial proportional log odds (Ananth and Kleinbaum 1997), respectively using SAS version 9.0 (Cary, North Carolina).

3. Results

Forty-three herd management variables were initially evaluated. By assessing variance inflation, it was determined that significant multi-collinearity existed. Once data were stratified and evaluated by herd type, it was apparent that multi-collinearity differed by herd type. Herd type as a variable was only statistically significant in univariable analysis for ampicillin MIC_{50}. In order to control multi-collinearity analysis was performed in two separate models of conventional herds and organic herds. Due to significant differences in management parameters (Geiger, Ruegg et al. 2003) a reduction in the number of herd management parameters assessed for organic herd MIC_{50} was performed. Twenty- eight herd

management variables were subsequently evaluated for organic herd MIC_{50} values for each antimicrobial.

3.1 Ceftriaxone

Conventional Farm MIC_{50}

Variables which were presented for multi-variable analysis for ceftriaxone included raw bacterial count, use of coccidiostats, rodent control, intramammary dry treatment, level of ceftiofur use, use of sulfa drugs, calf antibiotic exposure (milk replacer and grain), the type of lactating housing, the use of disinfectant in calf milk buckets, whether hutches were moved between calves, the use of animal proteins in the ration, an anionic transition cow diet and the level of sick calf treatments. Variables that were retained in the final model included the level of ceftiofur use, the exposure of calves to antibiotics in milk replacer and grain, the use of sulfa drugs to treat cattle and whether milk buckets and bottles were cleaned with a disinfectant (Table 14). Interestingly low use of ceftiofur was significantly associated with decreased odds (OR=0.3, 95% 0.1- 0.9) of reduced susceptibility to ceftriaxone compared to high level of use. However, there was not a statistically significant difference between no use of ceftiofur at all and high use (OR= 0.1 95% CI +0.1, 1.4) . There was a tendency for moderate use of ceftiofur to be associated with decreased susceptibility to ceftriaxone compared to high use of ceftiofur (OR = 1.4, 95% 0.4 – 5.9). Moderate exposure of calves to antibiotics was defined as either in milk replacer or grain. Whereas, high use was defined as calves exposed to antibiotics in both milk and grain. Farms with

moderate exposure of their calves to antibiotics were at increased odds for decreased susceptibility to ceftriaxone (OR= 4.0, 95% CI = 1.2 – 13.7). No use of any sulfa drugs to treat cattle resulted in lower odd of decreased susceptibility to ceftriaxone (OR=0.1, 95% CI= .02-.9). If farms did not sanitized the equipment used to feed milk, this resulted in increased odds for decreased susceptibility to ceftriaxone (OR= 3.3, 95% CI 1.2 – 9.6)

Table 14: Ceftriaxone Decreased Susceptibility – Final Multivariable Model for Conventional Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Ceftiofur Use	None vs. High	0.4	0.1	1.4
	Low vs. High	0.3	0.1	0.9
	Moderate vs. High	1.4	0.4	5.9
	High Use	1.0		
Calf AB Exposure	None vs. High	1.0	0.26	4.0
	Moderate vs. High	4.0	1.2	13.7
	High Exposure	1.0		
Sulfa Use	None vs. Use	0.1	.02	.9
	Yes	1.0		
Disinfect Milk	No	3.3	1.2	9.6
	Yes	1.0		

Organic Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included open herd status, the use of coccidiostats, a designated

sickpen, the use of a core antigen vaccine, the type of calf housing used, whether the lactating herd was grazed, the use of a disinfectant to clean calf milk buckets/bottles, and cross contamination of the feed by using the same loader tractor bucket to handle manure. Only the use of a coccidiostat and the presence of a designated sickpen were retained in the final model of cetriaxone susceptibility for organic farms (Table 15). If no coccidiostat was used, increased odds for decreased susceptibility to ceftriaxone was observed (OR=10.7, 95% CI 1.2-96). No designated sick pen was associated to lower odds of decreased susceptibility to ceftriaxone (OR=0.1, 95% CI = .01-.6)

Table 15: Ceftriaxone Decreased Susceptibility – Final Multivariable Model for Organic Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Coccidiostat Used	No	10.7	1.2	96
	Yes	1.0		
Sick Pen	No	0.1	.01	0.6
	Yes	1.0		

3.2 Sulfamethoxazole

Conventional Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included somatic cell count, milk production, the use of a tetracycline-sulfa crumble in the heifer grain, a designated sickpen, the use of florfenicol, the level

of calf exposure to antibiotics (milk replacer/grain), the housing of sick cows with cows due to calve, whether chlorine was added to cattle watering tanks, the exposure of cattle to surface water, and the level of sick cows treated on the farm. Somatic cell count, level of milk production, florfenicol use, maternity cows housed with sick cows, access to surface water, and the level of treated cows in the herd were retained in the final multi-variable model for conventional farms MIC_{50} (Table 16). The lowest SCC of less than 100,000 was associated with significantly lower odds of decreased susceptibility than farm with high SCC of greater than 400,000 (OR = 0.02, 95% CI .01-.9). The lowest two levels of milk production by quartile had significantly higher odds of decreased susceptibility to sulfamethoxazole compared to high producing herd (OR=19.4 and OR=5.1, respectively). If no florfenicol was used on the dairy, this resulted in lower odds of decreased susceptibility (OR=0.1, 95% CI= .03-.5). If sick cows were separated from fresh animals, lower odds of decreased susceptibility were observed (OR= 0.3 95% CI 0.1-0.9). Dairies that did not allow cows access to surface water had decreased odds of reduced susceptibility (OR= 0.3 95% CI 0.1-0.9). If either none of the mature herd or <10% had been treated, lower odds for decreased susceptibility were observed (OR= .06 and OR=.12, respectively).

Table 16: Sulfamethoxazole Decreased Susceptibility – Final Multivariable Model for Conventional Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
SCC	<100,000	.02	.01	1.9
	100,000-199,000	2.4	0.4	14.7
	200,000-299,000	1.0	0.2	5.3
	300,000-399,000	3.4	0.4	24
	400,000+	1.0		
Milk Qt	Lowest 25%	19.4	2.8	134
	26-50th Percentile	5.1	2.8	18
	50-75th Percentile	1.2	0.4	4.0
	Highest 25%	1.0		
Florfenicol Use	No	0.1	.03	.5
	Yes	1.0		
Maternity Housed with Sick Cows	None	0.3	0.1	0.9
	Yes	1.0		
Surface Water Access	None	0.3	0.1	.9
	Yes	1.0		
Level of Cow Herd Treated	None vs. Moderate	.06	.004	.9
	Low vs. Moderate	.12	.02	.6
	Moderate	1.0		

Organic Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included the duration the farm was had been organic, whether the lactating herd

grazed, the use of disinfectant to sanitized calf pens/hutches, the housing of sick cows with cows due to calve, the exposre of cattle to surface water, and the level of sick cows treated on the farm. Access to surface water and level of mature cows treated were retained in the final multivariable model for reduced susceptibility in organic herd MIC_{50} (Table 17). If cows were not allowed access to surface water, a significantly lower odds of reduced susceptibility was observed (OR=0.1 95% CI .01-0.7). However, if no cow treatments were reported in the prior 60 day, a remarkable increase in the odds of decreased susceptibility to sulfamethoxazole was detected (OR=20.4, 95% CI= 1.1-430)

Table 17: Sulfamethoxazole Decreased Susceptibility – Final Multivariable Model for Organic Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Surface Water Access	No	0.1	.01	.7
	Yes	1.0		
Cows Treated	No	20.4	1.0	430
	1-10% of Cows treated	1.0		

3.3 Kanamycin

Conventional Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included the use of a core antigen vaccine, feeding at least 3 quarts of colostrum, whether the lactating herd grazed, the type of lactating housing, the use of a transition ration, feeding an anionic ration to close –up cows, and cross contamination of the feed by using the same loader tractor bucket to handle

manure. The use of a core antigen vaccine, feeding less than 3 quarts of colostrum, whether dry cows were grazed and the use of a transition ration were retained in the final multivariable model for decreased herd susceptibility to kanamycin (Table 18). If a core antigen vaccine was not used a higher odds of decreased susceptibility was observed (OR=3.1 95% CI= 1.2 -8.4). Feeding less than 3 quarts of colostrum to newborn calves was also associated with increased odds of decreasing herd susceptibility (OR-5.8, 95% CI 1.0-39). However, keeping dry cows confined rather than grazing and not using a transition ration were associated with lower odds of decreased susceptibility (OR= 0.3 and OR=0.3, respectively)

Table 18: Kanamycin Decreased Susceptibility – Final Multivariable Model for Conventional Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Core Antigen Vaccine	Not used	3.1	1.2	8.4
	Used	1.0		
Colostrum Feeding	Feed 2 Quarts or less	5.8	1.0	39
	Feed 3-4 Quarts	1.0		
Dry Cow Grazing	Not grazed	0.3	0.1	0.7
	Dry Cows are Grazed	1.0		
Transition Ration	None	0.3	.08	.9
	Transition Ration Used	1.0		

Organic Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included whether the farm participated in DHIA (Dairy Herd Improvement Association), the use of coccidiostats, rodent control, the type of calf housing, whether a TMR (total mixed ration) was fed to the lactating herd, the use of a transition ration, and the level of sick calf treatments. The final multivariable model for organic farm susceptibility to kanamycin retained DHIA member status, the use of coccidiostats, calf housing and rodent control (Table 19). If organic farm were not enrolled in DHIA, a lower odds of decreased susceptibility to kanamycin was found (OR=0.5, 95% CI .03 - .9). No use of a coccidiostat and no use of rodent control both demonstrated much lower odds of decreased susceptibility (OR=.05 and .01, respectively). A very remarkable affect of calf exposure to other calves was found compared to calves isolated in hutches. Calf housed in group pens and calf barns were at very high odds for decreased susceptibility to kanamycin (OR=78, OR=37, respectively).

Table 19: Kanamycin Decreased Susceptibility – Final Multivariable Model for Organic Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
DHIA	Not used	0.5	.03	0.9
	Used	1.0		
Coccidiostat Use	None Used	0.05	.004	.8
	Coccidiostate Used	1.0		
Biosecurity of Calf Housing	Group Pens	78	1.3	999
	Calf barn	37	1.0	999
	Individual Hutches	1.0		
Rodent Control	None	.01	.001	.5
	Rodent control Used	1.0		

3.4 Tetracycline

Conventional Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included bacterial count of the milk, the use of a coccidiostat, a designated sickpen, the level of herd mortality, the level of ceftiofur use, the use of florfenicol, the type of lactating housing, whether calf hutches are moved in between calves, the use of chlorine to sanitized cattle watering tanks, and whether cattle are grazed where manure had been spread. Only the movement of hutches between calves was statistically significant (Table 20). If hutches are

not moved the odds of decreased susceptibility to tetracycline is increased (OR=4.0, 95% CI= 1.0 -17.6).

Table 20: Tetracycline Decreased Susceptibility – Final Multivariable Model for Conventional Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Hutch Management	Hutches are Not Moved	4.0	1.0	17.6
	Hutches moved between Calves	1.0		

Organic Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included the use of coccidiostats, whether calf hutches are moved in between calves, and the use of a transition ration. The multivariable model for organic farm MIC_{50} retained two variables, whether hutches were moved between calves and the use of a transition ration (Table 21) . If hutches were not moved between calves, a higher odds of decreased susceptibility resulted (OR 4.0, 95% CI 0.7-27). This variable was retained due to overall model fit, compared to a more reduced model. The use of a transition ration resulted in much lower odds of decreased susceptibility compare to no transition ration (OR=.08 95% CI 0.1-.7)

Table 21: Tetracycline Decreased Susceptibility – Final Multivariable Model for Organic Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Hutch Management	Hutches are Not Moved	4.0	0.7	27
	Hutches moved between Calves	1.0		
Transition Ration	Transition Ration Used	.08	.01	.7
	No Transition Ration	1.0		

3.5 Ampicillin

Conventional Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included the use of tetracycline-sulfa crumbles in the heifer grain, the level of ceftiofur use, the use of florfenicol, type of calf housing, type of lactating cow housing, whether at least 3 quarts of colostrum are fed to newborn calves, and the use of a transition ration. The final multi-variable model retained level of ceftiofur used and use of a tetracycline-sulf crumble (Table 22). Interestingly, moderate use of ceftiofur and no use ceftiofur resulting in significantly lower odds of decreased susceptibility to ampillin (OR=0.7 and 0.3, respectively). However, low use of ceftiofur was not significantly different from high use of this antimicrobial. If calves were not exposed to a transition grain crumble of tetracycline-sulf these herds were at lower odd for ampicillin herd MIC_{50} .

Table 22: Ampicillin Decreased Susceptibility – Final Multivariable Model for Conventional Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Ceftiofur Use	Not Used	.3	.18	.9
	Low	.9	.3	2.7
	Moderate	.7	.1	.9
	High level of Use	1.0		
AS Crumbles	Not Fed to Transition Calves	.2	.04	.7
	Fed to Transition Calves	1.0		

Organic Farm MIC_{50}

Variables that met the inclusion criteria for multivariable model development included open herd status, whether other animals are present on the farm, the presence of a designated sickpen, the type of calf housing, the type of lactating cow housing, and using a shared pen for sick cows and cows due to calve. For organic farm open herd status and use of a designated sick pen were both associated with lower odds of herd MIC_{50} (Table 23).

Table 23: Ampicillin Decreased Susceptibility – Final Multivariable Model for Organic Herds

Variable	Level	Odds Ratio	95% Confidence Interval	
			Lower Bound	Upper Bound
Herd Status	Open Herd	.1	.03	.5
	Closed Herd	1.0		
Sick Pen	None Designated	.2	.04	.9
	Dedicated Sick Pen			

As previously stated all herd MIC_{50} values for ciprofloxacin were 0.5 ug/ml; therefore, no assessment of herd management risk factors could be made.

4. Discussion

Particular strengths of this study included the number and diversity of farms enrolled, the longitudinal sampling design, and the number of parameter summarized through herd questionnaires. Rather than only assess antimicrobials of interest in human medicine, the inclusion of antimicrobial used on dairies allowed plausible exposures to be evaluated. By retaining MIC values as outcomes for isolates from farms, an MIC_{50} could be calculated to represent the farm outcome. By maintaining an ordinal outcome, more subtle differences in susceptibility can be evaluated using GEE methodology of proportional odds and partial proportional odds models.

While some studies have evaluated antimicrobial susceptibility in food animals by farm type and presumed antibiotic exposures(Mathew, Beckmann et al. 2001; Sato, Bartlett et al. 2004), this is the first study known to assess this level of detail in farm management practices which may impact the ecology of *Campylobacter* resistance determinants.

Some consistencies can be noted across the different antimicrobials. Measures of hygiene management on farms were found to be significant in several models. Cleaning milk buckets or bottles, use of a separate sick pen, keeping animals due to calve away from sick cows, low somatic cell counts, raising calves in individual hutches, and moving hutches between calves were found to reduce the odds of higher farm MIC_{50} to antimicrobials. In some of the models. exposure to an antimicrobial may impact the susceptibility of the *Campylobacter* on a given farm. The use of a transition calf rations which incorporate a tetracycline-sulfa crumble, the use of florfenicol, levels of ceftiofur used, the use of coccidiostats, and the amount of antibiotics calves are exposed to in milk and grain. However, there is not a clear trend of more exposure equating to higher MIC_{50}, as was observed with calf antibiotic exposure and level of ceftiofur used where the more moderate exposure had higher odds of decreased susceptibility. Two parameters of "immune support" also were associated with the lower herd MIC_{50} levels, feeding adequate colostrum and the use of a core antigen vaccine. The impact of potential fecal contamination from other cows or wildlife and subsequent exchange of enteric bacteria or determinants of resistance may be associated to the variable of surface water access and grazing of differing animal

groups. Indeed, antimicrobial resistance has been documented in wild birds and may be a source to cattle that are turned out (Stanley and Jones 1998). The strength of the milk quality parameters and level of production were remarkable in the conventional herd model for sulfamethoxazole, which may demonstrate that the intensive management required for high milk production is not associated with antimicrobial resistance in *Campylobacter* in dairy cattle.

Few of the above findings can be compared to other literature on the subject of antimicrobial susceptibility due to limited assessment of farm management practices. Sato et al., 2004 did not find significant difference between Campylobacter susceptibility from organic and conventional farms for ciprofloxacin, gentamicin, erythromycin or tetracycline (Sato, Bartlett et al. 2004). Similarly, our study found that the only antimicrobial where herd type was significant on univariable analysis was for the MIC_{50} for ampicillin. A study in the UK could not find an association between on farm antimicrobial use and subsequent susceptibility patterns in *Campylobacter* to the drug of interest (Piddock, Ricci et al. 2000). Recently the antimicrobial surveillance system in Denmark reported an interesting finding that supports some of the finding presented here. While many variables for antimicrobial use and exposure on dairy farms were assessed in this study, few were found significant in final models. Similarly, DANMAP reported that due to outbreaks of post-weaning multi-systemic wasting syndrome there was a large increase in the use of tetracycline in pork production in Denmark. However, no increase in antimicrobial resistance was observed in indicator bacteria (Heuer and Larsen

2004) . Therefore, some of the assumptions that use necessarily results in decreased susceptibility may not hold up to closer scrutiny.

However, as we demonstrated above, often associations are found between other antimicrobial exposures on the farm and decreased susceptibility in our *Campylobacter* isolates. For example with ampicillin MIC_{50}, beta-lactam use was not significant; while ceftiofur use was retained in the final model. Both beta-lactams (such as penicillin or ampicillin) and ceftiofur were commonly used on dairies in this study (Geiger, Ruegg et al. 2003). Also florfenicol use was associated with higher farm MIC_{50} for sulfamethoxazole. These findings of "other" selective pressure than perhaps the main exposure considered, are supported by other authors. Avrain et al., in 2003 had found associations with tetracycline resistance in broiler chickens to not only be associated to flocks treated with this drug, but also with birds that had been exposed to a coccidiostat only (Avrain, Humbert et al. 2003). Coccidostats are frequently used in conventional dairy heifer rations, but this was not a common practice in our organic herds (Geiger, Ruegg et al. 2003).

It is unclear if some common mechanism for phenotypic decreased susceptibility may be turned on as with efflux genes with the selective pressure of another antimicrobial (Lin, O. et al. 2002; Pumbwe, Randall et al. 2004). Also it is known that both tetracycline and kanamycin genetic determinants are carried on plasmids in *Campylobacter* and often resistance to both antimicrobials is co-associated (Kotarski, Merriwether et al. 1986; Taylor 1986; Tenover, Fennell et

al. 1992). Recently the *pTet* plasmid from *Campylobacter* has been sequenced and similarities to the *pVir* plasmid were identified (Batchelor, Pearson et al. 2003). It may be that tetracycline and kanamycin resistance persist in *Campylobacter* from dairy animals without an associated exposure due to the fitness virulence genes also carried on the plasmid confer to the particular strain of *Campylobacter* .

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5. Conclusion

With the exception of ampicillin decreased susceptibility was not associated to farm type. Also exposures to other antimicrobials appear to be associated to increasing MIC in some antimicrobials. The findings here reinforce sound practices of husbandry and animal housing in reducing cross contamination between animal housing facilities and maintaining clean feeding equipment. Also the role of cattle exposure to wildlife through grazing and surface water may warrant further investigation due to the associations of decreased susceptibility in some antimicrobials. Both our study findings and trends observed globally demonstrate that the issue of antimicrobial resistance in food animals is complex and warrants continued investigation to insure both food safety and a healthy livestock population.

CHAPTER FIVE

Genetic mechanisms contributing to reduced tetracycline susceptibility of *Campylobacter* isolated from organic and conventional dairy farms in the Midwestern and Northeastern United States

ABSTRACT

Campylobacter is one of the most common causes of gastroenteritis and can be acquired through contact with farm animals or the consumption of raw milk. Since there are concerns over the role of food producing animals in the dissemination of antimicrobial resistance to humans, we evaluated the prevalence of antimicrobial resistance in *Campylobacter* isolates from dairy farms and the genetic mechanism conferring the observed resistance. Evaluation of antimicrobial resistance was completed on 912 isolates from conventional and 304 organic dairy farms to 8 drugs (azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid and tetracycline) using mirobroth dilution. Resistance to 7 of 8 drugs was very low and did not differ by farm type. However, tetracycline resistance was common to *Campylobacter* isolated from both organic and conventional dairy farms, 48% and 58% of isolates respectively. We identified that tetracycline resistance in both farm types was highly associated to the carriage of *tetO* in *Campylobacter* isolates through mulit-plex PCR (X^2 = 124, p <0.01) and that the agreement between phenotypic

tetracycline resistance and the genetic determination of resistance was quite good (Kappa = 0.86)

Introduction

Campylobacter is the most frequently identified cause of bacterial gastroenteritis in the United States. (Acheson 2001) (Altekruse and Tollefson 2003) Most *Campylobacter* enteritis cases are mild, self limiting episodes of vomiting, cramping and diarrhea (Tauxe, Hargrett-Bean et al. 1988) (Altekruse, Swerdlow et al. 1998) However, a more serious form of the illness occurs in infants, geriatric patients, and immune suppressed individuals requiring antimicrobial therapy. (Blaser 1997)

Another primary concern is that *Campylobacter* isolates are displaying increased resistance to many classes of antimicrobial agents throughout time. (Neu 1992) (Engberg, Aarestrup et al. 2000) Globally the patterns of antimicrobial resistance in *Campylobacter* differ by country of origin. Overall susceptible strains of *Campylobacter* are identified in Scandinavian countries where antimicrobial use is highly regulated. Surveillance data of susceptibility of Campylobacter through the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) demonstrate low levels of resistance of *C. jejuni* to fluoroquinolones, erythromycin, tetracycline (<6%, <6%,<11%, respectively) (Aarestrup, Nielsen et al. 1997). Countries where antimicrobial use is less regulated, such as Spain and Thailand, tend to observe high levels of resistance

to many classes of antimicrobials (Saenz, Zarazaga et al. 2000; Padungtod and Kaneene 2003).

Many researchers have focused on the development of resistance to fluoroquinolones in both food animals such as poultry and also in human isolates of *Campylobacter*(Smith, Besser et al. 1999) (Engberg, Aarestrup et al. 2000; Nackamkin, Ung et al. 2002). The mechanism of resistance to this class of compounds is chromosomally mediated by mutations within the *gyrA* and/or *parC* gene (Wilson, Abner et al. 2000; Padungtod and Kaneene 2003; Piddock, Ricci et al. 2003). Macrolide resistance, to such drugs as erythromycin, has also been identified as a chromosomally mediated event in *Campylobacter* which results in alteration of the ribosome (Engberg, Aarestrup et al. 2000). However, tetracycline resistance in *Campylobacter* has been identified to be linked to the gene *tetO* which is typically associated with a large plasmid (Taylor 1986; Lee, Tai et al. 1994). This location of resistance allows not only clonal expansion of tetracycline resistance as plasmids are copied and partitioned during cell division, but also the potential for horizontal movement of resistance genes through transmissible plasmids (Taylor, DeGrandis et al. 1981).

Increased fluoroquinolone resistance in *Campylobacter* and other bacteria has been documented by some investigators once these antimicrobials were approved in some food animal species.(Smith, Besser et al. 1999; McDermott, Bodeis et al. 2002) However, there has also been evidence of increased susceptibility in bacteria when certain antimicrobials were banned from

use.(Aarestrup, Seyfarth et al. 2001; Boerlin, Wissing et al. 2001) However, most of literature has relied upon ecological studies where exposure is assumed for the group animals (i.e. which drugs are approved for veterinary use in a particular country) without ascertaining actual exposure to the drugs being studied and has focused on poultry or human isolates (Harris, Thompson et al. 1986; Jacob-Reitsma, Koenraad et al. 1994) (Smith, Besser et al. 1999; Nackamkin, Ung et al. 2002) These kinds of studies are useful, but they are prone to ecological fallacy. Most research on *Campylobacter* resistance has been on drug classes such as fluoroquinolones and macrolides, but it is important to note that these antimicrobials are not used on dairy farms (Hady, Lloyd et al. 1993; Sundlof, Kaneene et al. 1995). It has been established that healthy adult cows and calves can frequently shed this organism in their manure. (Wesley, Wells et al. 2000; Green, Kaneene et al. 2001) (Nielsen 2002) Moreover, a number of outbreaks of *Campylobacter* enteritis have been associated with raw milk consumption (Kalman, Szollosi et al. 2000) (Warner, Bryner et al. 1986; Dilworth, Lior et al. 1988; Lehner, Schneck et al. 2000), dairy farm visits(Evans, Roberts et al. 1996) , and water contamination (Duke, Breathnach et al. 1996) (Melby, Svendby et al. 2000; Frost, Gillespie et al. 2002). Therefore, the dairy industry must be examined for the role it may play in contributing this foodborne pathogen and potential route of antimicrobial resistance to human food and water sources.

The objectives of this study were to 1) determine if antimicrobial resistance in *Campylobacter* varies by farms with known antimicrobial use and animal exposure by comparing isolates from organic and conventional dairy farms 2) to identify the mechanism of resistance of antimicrobial(s) with significant resistance on dairy farms 3) to determine whether the mechanism of resistance is similar in the two farm types and across phenotypic expression of resistance by a range of minimum inhibitory concentration (MIC) values for that drug.

MATERIALS AND METHODS

Source of *Campylobacter* Isolates: *Campylobacter spp.* from were isolated from 128 organic and conventional dairy farms in Michigan, Minnesota, New York, and Wisconsin. Isolates include *Campylobacter* from cattle representing the different animal management groups on the dairies and also *Campylobacter* isolated from the farm environment.

***Campylobacter spp.* Isolation and Identification:** Environmental swabs and milk filter were enriched in Bolton broth (Oxoid) containing 5% laked horse blood and selective antimicrobial agents (20mg/L cefaperazone, 20 mg/L vancomycin, 20 mg/L trimethoprim, 50 mg/L cycloheximide). The enriched samples were then incubated at 42° C in 5-10% CO_2 for 48 hours. Animal fecal samples and milk samples were suspended in phosphate buffer saline solution. PBS suspended biological samples and enriched samples were streaked on selective *Campylobacter* Blaser plates (BD Diagnostics,) and incubated at 42° C

in 5-10% CO_2 for 48 hours. Typical colonies were selected and streaked on sheep blood agar (SBA) and incubated at 42°C in 5-10% CO_2 for 48 hours. *Campylobacter* identification was performed from isolated colonies by gram staining, oxidase testing, and motility testing. Hippurate hydrolysis was used to speciate *C. jejuni*. Over 97% of our isolates were classified as *C. jejuni*. (Green, Kaneene et al. 2001)

***In vitro* susceptibility testing –Microbroth Dilution.**

In vitro susceptibility testing was performed using the microbroth dilution method, following guidelines provided by the National Committee on Clinical Laboratory Standards (NCCLS) (NCCLS 2003). Antimicrobial susceptibility was performed for 912 *Campylobacter* isolates from conventional dairy farms and 304 *Campylobacter* isolates from organic dairy farms. Bacterial isolates from frozen stock were grown on Brucella agar supplemented with 5% defribrinated sheep blood (BASB) for 48 hours at 42°C under microaerophilic conditions. Individual colonies from each plate were subcultured on BASB under similar growth conditions. Bacteria were swabbed from the BASB and suspended in 5 ml H_2O and the turbidity was adjusted to a 0.5 McFarland standard. This suspension was used to make a 1:10 dilution into *Haemophilus* testing medium (HTM), resulting in a final bacterial inoculum concentration of approximately 8×10^5 CFU/ml.

Customized microbroth dilution plates (CMV1USDA) were purchased pre-made from TREK Diagnostic Systems, Inc. (West Lake, Ohio USA), with a

prepared range of drug concentrations of azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid, and tetracycline. *C. jejuni* ATCC33560 and 81176 were used as quality control strains. Each plate was inoculated by adding 100 ul of the bacterial suspension using a Sensititre autoinoculator, covered with a gas-permeable seal, and incubated at 42°C in microaerophilic conditions for 48 hours. The minimum inhibitory concentration (MIC) was determined as the minimum antimicrobial dilution at which no bacterial growth occurred.

The breakpoints used to categorize isolates as resistant or not resistant were those recommended by the National Antimicrobial Resistance Monitoring System (NARMS) (Table 24).

Table 24: Dilution ranges for the antimicrobial agents and interpretative breakpoints

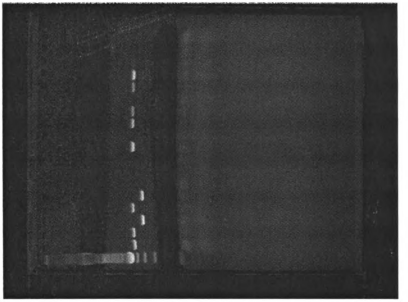
Antimicrobial Agent	Microbroth Dilution Test Ranges (ug/ml)	NARMS Interpretative Breakpoints for Resistant
Azithromycin	0.03 - 256	≥ 2
Chloramphenicol	0.5 - 64	≥ 32
Clindamycin	0.06 - 256	≥ 4
Ciprofloxacin	0.03 – 64	≥ 4
Eythromycin	0.12 - 256	≥ 8
Gentamicin	0.12 - 256	≥ 16
Nalidixic Acid	0.12 – 128	≥ 32
Tetracycline	0.25 - 256	<=4 8 >=16 [d]

Identification of genetic determinants for antimicrobial resistance:

Isolates used for PCR determination of tetracycline resistance markers included 167 isolates, comprised of 128 from conventional and 39 from organic dairy farms. Isolates from both farm types included a range of MICs to tetracycline from 0.25 ug/ml to 256 ug/ml. These isolates demonstrated consistent MICs through multiple regrowths from freezer stock. A modification in the multiplex PCR procedure used by Ng et al., was used to identify genetic markers in tetracycline resistant isolates. (Ng, Martin et al. 2001) Briefly, 50 ug of reaction mix was prepared using 0.5 ug template DNA, 1 x PCT buffer, 2.5 U DNA Taq polymerase (Perkin-Elmer, Norwalk CT, USA), 300uM each of dATP, dCTP, dGTP, dTTP (Perkin-Elmer), and ddH_2O (Ng, Martin et al. 2001). Since most literature has identified *tetO* as being associated to tetracycline resistance in Campylobacter (Lee, Langlois et al. 1993; Randall, Ridley et al. 2003) and homology between *tetO* and t*etM* and both confer ribosomal protection (Levy, McMurry et al. 1999; Chopra and Roberts 2001) and have been found in rumen flora (Aminov, Garrigues-JeanJean et al. 2001) , 1.25 uM and 0.5 uM of primers for *tetO* and *tetM*, respectively, were included in the reaction mix (Ng, Martin et al. 2001). Thermocycler conditions for amplification were 5 minutes at 94 ˚ C for initial denaturation, followed by 35 cycles of 94 ˚ C for 1 min, 55 ˚ C for 1 min, and 72 C for 1. 5 min (Ng, Martin et al. 2001). PCR products were analyzed via gel electrophoresis, using a 110 bp ladder and visualized by ethidium bromide staining and U.V. transillumination. Strains used for quality control include

Campylobacter 81176, 33560, and *E coli* with pJ13 (*tetM*) and pOUA1 (*tetO*) kindly provided by Ng's research team (Figure 3).

Figure 3. Multiplex PCR for Tet Determinants



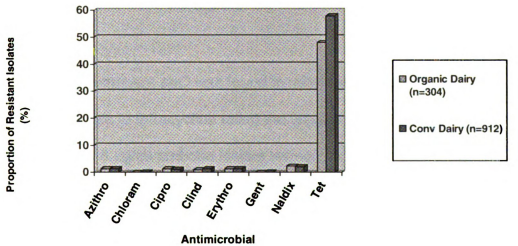
Lane 1: 100 bp ladder; Lane 2 & 3: Ecoli with pOUA1 (tet O); Lane 4 & 6; E coli with pJ13 (tet M); Lane 5: 81176; Lane 6 & 7: 33560; Lane 8: Conv Isolate MIC =0.5; Lane 9: Conv. Isolate MIC = 16; Lane 10:
Organic Isolate MIC=2; Lane 11: Organic Isolate MIC=128; Lane 12: Organic Isolate MIC=32;
Lane 13: Conv Isolate MIC=0.25; Lane 14: Conv Isolae MIC =16; Lane 15: Conv Isolate MIC=128:
Lane 16 & 17: Blank control

Data analysis: Chi-Square testing was performed to identify the association between farm type and antimicrobial resistance and to test the association between the isolates carrying *tetO* and tetracycline resistance. A Kappa value was calculated for the agreement between carriage of *tetO* and the phenotypic expression of Tetracycline resistance, using SAS Version 8.2 Cary, North Carolina.

RESULTS

Using the 8 antimicrobials being evaluated by NARMS, *Campylobacter* isolates from both organic and conventional dairies demonstrated very low levels of resistance to 7 of the 8 drugs. (Figure 3.) There was no statistical difference ($p > 0.05$) between resistance in *Campylobacter* isolates from organic dairy farms and conventional dairy farms for azithromycin, chloramphenicol, ciprofloxacin, clindamycin, enrofloxacin, gentamicin, and nalidixic acid. Tetracycline resistance was common in *Campylobacter* isolated from both organic and conventional farm types, 48% and 58%, respectively. Resistance to tetracycline was significantly higher on conventional than organic dairy farms. ($p < 0.01$).

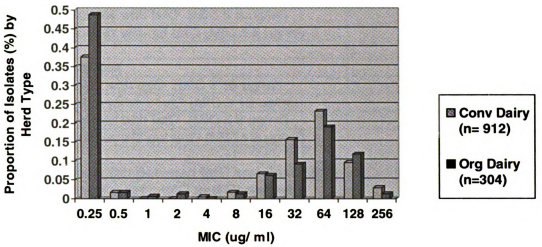
Figure 4. Prevalence of Resistant Isolates by Farm Type



Tetracycline MICs demonstrated a bimodal distribution in both herd types (Figure 4). *Campylobacter* from both conventional dairy farms and organic dairy farms had the largest proportion of isolates (37.6% and 48.6%, respectively) with very susceptible MICs to tetracycline of ≤ 0.25 ug/ml. The proportion of highly

susceptible isolates (MIC ≤ 0.25 ug/ml) was significantly higher on organic farms ($p < 0.05$).

Figure 5. Distribution of Isolates by Tetracycline MIC



The genetic determinant *tetO* was found in nearly all *Campylobacter* demonstrating resistance to tetracycline using the breakpoint of 16 ug/ml. Nine isolates with an MIC of 8 ug/ml and one isolate with an MIC of 4 ug/ml were found to carry *tetO*. No isolates which had been determined to be susceptibly to tetracycline were found to have either *tetO* or *tetM*. No *tetM* was identified in any of our resistant isolates. Three isolates which had been determined to be resistant to tetracycline did not demonstrate *tetO*. These isolates were retested for susceptibility to tetracycline using the Kirby Bauer method and had become susceptible to this antimicrobial following serial passage needed to regrow

isolates from frozen stock. These three isolates were excluded from the analysis (Table 25).

Table 25. Proportion of *Campylobacter spp.* Isolates with *tetO* and Resistance

Variable	Value
Conventional Dairy Isolates	128
Resistant Isolates with tetO (MIC ≥ 16 ug/ml)	75
Resistant Isolates without tetO (MIC ≥ 16 ug/ml) *	3*
Susceptible Isolates with tetO (MIC ≤ 8 ug/ml)	7
Susceptible Isolates without tetO (MIC ≤ 8 ug/ml)	43
Organic Dairy Isolates	39
Resistant Isolates with tetO (MIC ≥ 16 ug/ml)	23
Resistant Isolates without tetO (MIC ≥ 16 ug/ml)	0
Susceptible Isolates with tetO (MIC ≤ 8 ug/ml)	3
Susceptible Isolates without tetO (MIC ≤ 8 ug/ml)	13
Chi Square Association Between tetO and Resistance	$X^2 = 124$ ($P < 0.001$)
Kappa Agreement Between tetO and Resistance	0.86

* These three isolates were retested with Kirby-Bauer disk diffusion and had become susceptible to Tetracycline.

Overall, the association between tetracycline resistance and the carriage of *tetO* was highly significant ($X^2 = 124$, $p < 0.001$). The agreement between identification of tetracycline resistance by phenotypic methods (MIC determination) and genetic methods (PCR for *tetO*) was very good (Kappa = 0.86).

Discussion

Overall our research agrees with authors who have studied farming systems with more regulated drug use such as the Scandinavian countries

(Aarestrup, Nielsen et al. 1997). However, enrofloxacin and nalidixic acid resistance in cattle isolates from the same study was higher (3 % and 14%, respectively) in Denmark than we reported in our dairy isolates from four states in the upper Great Lakes region of the United States. Since the study by Aarestrup and co-workers in 2000 study included cattle from slaughter, enrofloxacin may have been used in the treatment of beef cattle since this drug was approved for veterinary use in 1993 in Denmark. In the United States, fluoroquinolone use in dairy cattle is strictly prohibited. In 2001 erythromycin resistance in *Campylobacter* isolated from cattle was 8% (DANMAP 2001). However in 2002 none of the 53 *C. jejuni* isolated from cattle was resistant to this drug. (Emborg and Heuer 2002) Overall lower resistance to tetracycline (6%) was observed in campylobacter isolated from cattle in Denmark (Aarestrup, Nielsen et al. 1997) (DANMAP 2001) than what was found in our dairy isolates. It should be noted that the Danish work includes slaughter cattle and is not focused on dairy animals. Therefore, the animal type and husbandry may not be comparable. Also the sample size of *C. jejuni* from cattle is much smaller in the Danish survey (n=53) than in the work presented here (n =1216).

Piddock and colleagues (Piddock, Ricci et al. 2000) evaluated *Campylobacter* susceptibility to five antimicrobials on primarily dairy farms in the United Kingdom. The study by Piddock an co-workers was one of few which ascertained both farm use of antimicrobials and some individual animal treatments. Interestingly, Piddock and colleagues found no clear associations between on-farm antimicrobial use and susceptibility patterns in *Campylobacter*

isolates to tetracycline, kanamycin, ciprofloxacin, erythromycin, or nalidixic acid (Piddock, Ricci et al. 2000).

In our study we demonstrated that resistance to tetracycline was significantly higher on conventional farms than organic farms. It is not surprising that there is resistance to tetracycline on conventional farms, since this antimicrobial is often used in calf milk replacer, some calf grain, and also is used to therapeutically treat ill animals on conventional dairy farms (Geiger, Ruegg et al. 2003). It was interesting to find that tetracycline resistance was still common in 48% of the *Campylobacter* isolated on organic dairy farms despite only 1 of the 32 organic farms using tetracycline in milk replacer and none of the organic farms using tetracycline therapeutically (Geiger, Ruegg et al. 2003).

Tetracycline resistance in Campylobacter isolated from outbreaks of various sources. One milk borne outbreak of Campylobacterosis which occurred in Arizona in 1981 was found to be tetracycline resistant (Bopp, Birkness et al. 1985). However, isolates from two other milk borne outbreaks were not classified as having tetracycline resistance, and were in fact susceptible to most drugs tested (Bopp, Birkness et al. 1985). These authors found that tetracycline resistance was associated with a 38 mega-dalton plasmid; but genetic markers were not described (Bopp, Birkness et al. 1985)

Our findings agree with those of Aminov et al.,(Aminov, Garrigues-JeanJean et al. 2001) Their research found *tetO* was in the rumen of cattle which had not received tetracycline either in feed or for therapeutic reasons. However,

tetO and *tetM* were found in sows which received both prophylactic antimicrobials and tetracycline had been used to treat animals in the swine facility. (Aminov, Garrigues-JeanJean et al. 2001) While in the study by Aminov et al (2001) molecular markers for tetracycline resistance in all gastrointenstinal flora were identified, so that the level of *tetO* in *Campylobacter* was not specifically determined (Aminov, Garrigues-JeanJean et al. 2001). Interestingly, Aminov and colleagues also identified genetic markers for tetracycline resistance in swine feed (Aminov, Garrigues-JeanJean et al. 2001)

The level of tetracycline resistance which was found in isolates from either farm type in our study is higher than what other authors found in cattle in Northeast Portugal (Cabrita, Rodrigues et al. 1992). The prevalence of tetracycline resistance in cattle was 6.2%. Plasmids were detected in 18.0% of *C. jejuni* isolated from cattle. Since *tetO* was not evaluated by these authors, the plasmids identified may not have carried this genetic marker for tetracycline resistance (Cabrita, Rodrigues et al. 1992). Additionally, antimicrobial exposure of the animals was not ascertained and the number of campylobacter analyzed from cow samples was 32. Interestingly, Cabrita found both high prevalence of antimicrobial resistance and proportion of isolates carrying plasmids in *Campylobacter* isolated from rats (Cabrita, Rodrigues et al. 1992).

In 2002, Aquino and colleagues evaluated *Campylobacter* antimicrobial resistance and plasmid profiles in human and animal isolates. (Aquino, Filgueiras et al. 2002) The level of tetracycline resistance was 13.6%, which is much lower than in our study; and only one *C. jejuni* was identified as tetracycline resistant

(Aquino, Filgueiras et al. 2002). Number of isolates studied included 44 *Campylobacter*, 15 from humans, 9 from swine, 6 from sheep, 2 from poultry, 9 from rhesus monkeys, and 3 from dogs. The inclusion of research monkeys makes the overall findings difficult to evaluate, since a very high level of multiply resistant isolates and the carriage of plasmids were found in this population, compared to other species being evaluated (Aquino, Filgueiras et al. 2002). These authors did not evaluate genetic marker for tetracycline resistance nor the association between the carriage of plasmids and tetracycline resistant isolates (Aquino, Filgueiras et al. 2002).

Blake et al., in 2003 found that tetracycline exposure influenced the carriage of tetracycline resistant genes in general E coli. (Blake, Humphry et al. 2003) Similar to our findings in *Campylobacter*, this study found 82% of the intensively raised pig isolates were resistant to tetracycline. Resistance was also common in antibiotic free pig isolates with 62% demonstrating resistance (Blake, Humphry et al. 2003) The use of tetracycline in the intensive farms was not ascertained in either of their studies (Blake, Humphry et al. 2003). Also the number of organic or antibiotic free animals was very small, 3 pigs and 1 heifer. The number of intensively raised pigs that were sampled was 20; however, 10 E coli were characterized for each pig. Also in contrast to our work, the tetracycline markers evaluated were those involved in efflux, not ribosomal protection (Blake, Humphry et al. 2003).

While the mechanism of tetracycline resistance in *Campylobacter* has been well-described (Chopra and Roberts 2001; Spahn, Blaha et al. 2001)and documented in human (Bopp, Birkness et al. 1985) (Taylor, Chang et al. 1986), and poultry isolates (Lee, Tai et al. 1994) our study is the only known work evaluating the mechanism of resistance in *Campylobacter* from dairy farms in the United States.

Tetracycline's mode of activity is to block protein synthesis by stopping the elongation by interfering with the A-binding site on the ribosome (Connell, Trieber et al. 2003). The protein TetO is a ribosomal protection protein which acts directly with tetracycline and the 70s ribosome to cause the release of tetracycline (Connell, Trieber et al. 2003). It binds in a manner similar to Ef-G which is GTPase dependent (Manavathu, Fernandez et al. 1990). It has been suggested that Tet O causes conformational changes which persist in the ribosome even after Tet O is no longer bound (Connell, Trieber et al. 2003).

As opposed to genetic sequences considered to be "gram-negative" with high G + C content (>40%), t*etO* has a G+C content of <35% which is typical of gram positive nucleotide distributions (Chopra and Roberts 2001). Because of the level of homology between *tetM* and *tetO*, it has been hypothesized that Campylobacter acquired *tetO* from *Streptococcal sp* (Taylor 1986). Indeed *tetO* has been identified in *Streptococcus pneumonia* (Widdowson, Klugman et al. 1996). The two genetic sequences of *tetM* and *tetO* are 78% similar (Taylor 1986; Chopra and Roberts 2001). It should be noted that the current convention is to consider genetic sequences as the same gene if the amino acid sequences

is ≥80% in common (Levy, McMurry et al. 1999). For this reason, we felt it was important to ascertain the specificity of our primers to identify either *tetO* or *tetM* in our tetracycline-resistant campylobacter isolates. Only two genera of gram negative bacteria carry *tetO*, *Campylobacter* and the rumen bacteria *Butyrivibrio fibrisolvens* (Chopra and Roberts 2001) (Aminov, Garrigues-JeanJean et al. 2001). Our finding of only *tetO* and not *tetM* being associated to tetracycline resistance in *Campylobacter* is consistent with the findings of Randall et al., Using a breakpoint of 8 ug/ml for tetracycline, these researchers for 76% of their resistance strains carried *tetO*, but no *tetM* was detected in *Campylobacter* isolated from human, poultry and swine isolates (Randall, Ridley et al. 2003).

The three isolates which were classified as resistant, but were PCR-negative for genetic markers tetracycline resistance were an interesting finding in this study. When re-tested for antimicrobial susceptibility by disk diffusion, these isolates were re-classified as susceptible to tetracycline. One isolate was from an organic farm with an original MIC of 128 ug/ml. Two of the isolates were from conventional dairy farms and had with MICs of 32 ug/ml. Taylor et al., had described isolates becoming susceptible following laboratory handling (Taylor, Chang et al. 1986). This research group identified that these isolates were indeed plasmid free once they demonstrated susceptibility to tetracycline (Taylor, Chang et al. 1986). It was not noted what the original MIC of these isolates were. Presumably cured of the plasmid pTet which carries *tetO*.

Tetracycline resistance has been shown to be associated with a transmissible plasmid in *Campylobacter* (Taylor, DeGrandis et al. 1981) (Lee, Tai

et al. 1994; Velazquez, Jimenez et al. 1995). However, this genetic determinant may not be compatible with other gram negative bacteria which cause food poisoning such as *E. coli* or *Salmonella* (Taylor 1986). Because several Lee et al in 1994 and Pratt and colleagues in 2003 have also identified this markers for tetracycline resistance on chromosomal DNA in *Campylobacter,* and it is unclear if chromosomal *tetO* is is part of a mobile genetic element.

It would be informative to evaluate the basis of the differing phenotypic expressions of the same genetic determinant, as we have demonstrated that isolates with MICs from 8-256 carrying *tetO.* This is consistent with other authors who have found *Campylobacter* isolates with a range of MIC values to tetracycline all displayed similar outcomes when studied with molecular methods (Taylor, Chang et al. 1986) Copy number, either of the gene within the plasmid or copies of plasmid within a particular *Campylobacter* isolate, could account for differing MIC levels being expressed. However, there has been a significant amount of study focused on an upstream sequence from *tetO* that appears to be regulatory (Roberts 1996). This sequence is required for full expression of tetracycline resistance in isolates carrying *tetO* (Wang and Taylor 1991). Indeed, mutations in the DNA adjacent to *tetO* have been shown to affect the level of resistance to tetracycline, resulting in differing MICs being expressed (Wang and Taylor 1991) (Taylor, Trieber et al. 1998).

The diversity of MIC ranges displayed by our isolates with *tetO* could also be due to synergism with other mechanism of resistance in *Campylobacter* such

as an efflux system (Lin, O. et al. 2002). The expression of efflux pumps is controlled by regulatory proteins and expression of very high MIC to tetracycline may be due to overexpression of these regulatory proteins (Lin, O. et al. 2002). Indeed Lin and colleagues identified that *CmeABC* functions as a multidrug efflux system that can increase the expression of resistance to tetracycline by as much as an 8-fold increase in *Campylobacter* 81-176 (Lin, O. et al. 2002). The question remains why this efflux system would be induced in isolates from organic farms in the absence of selective pressure. Luo et al.,in 2003 documented that the *CmeABC* efflux pump is induced under selective pressure such as exposure to enrofloxacin (Luo, Sahin et al. 2003). Since dairy farms do not use any drugs from the fluoroquinolone class of antimicrobials, it would warrant further research to determine if other compounds on dairies (whether simple sanitizers or disinfectants) exert similar selective pressure for *Campylobacter* efflux systems to be expressed in vivo (Luo, Sahin et al. 2003).

Recently the plasmid, pTet, which carries *tetO* in *Campylobacter* was characterized (Batchelor, Pearson et al. 2003). Homology with pTet and pVir was found across many regions including type IV secretory systems and oriT regions encoding for plasmid transfer (Batchelor, Pearson et al. 2003). It may be that the presence of type IV secretory systems in pTet offer an advantage so that strains of *Campylobacter* carrying pTet do not become cured of the plasmid when there is an absence of antimicrobial pressure. It is possible that this is why

we still documented tetracycline resistance commonly in *Campylobacter* isolates from organic dairy farms what do not use tetracycline.

In summary, our research has demonstrated that *Campylobacter* from dairy farms in the United States is generally susceptible to most antimicrobials. However, tetracycline resistance was common in both organic and conventional dairies, although the level of resistance was significantly higher on conventional farms. We found that the carriage of *tetO* was highly associated to the phenotypic expression of tetracycline resistance in our isolates. Clear reasons of the maintenance of *tetO* in the absence of selective pressure on organic farms, and evaluation of specific risk factors on conventional dairy farm warrants further research.

DISCUSSION AND CONCLUSIONS

Campylobacter spp are the most common cause of bacterial gastroenteritis in many countries around the world. Outbreaks of Campylobacteriosis have been most notably attributed to the consumption of contaminated poultry, raw milk, educational visits to farms, and or can be waterborne. Recently there has been much concern about the documented occurrence of antimicrobial resistance in human *Camploybacter* cases. Since many human cases are acquired via the foodborne or waterborne route, it is prudent to examine food animal production systems which may contribute to the selection of resistance genes in this organism which may either contaminate food products or water through the application of animal manure. *Campylobacter* from dairy sources is very infrequently assessed as to its antimicrobial susceptibility profile despite human cases being attributed to raw milk, educational farm visits, and the potential for dairy cattle manure to contaminate water or other environmental sources.

Therefore, this study was developed with the overall goal of identifying risk factors hat may be explored as possible points of intervention to lessen antimicrobial resistance in *Campylobacter* in dairy cattle. This overall goal was addressed through the four following objectives: 1) Compare the patterns of antimicrobial resistance between organic and conventional dairy farm management types 2) Determine individual animal risk factors for decreased susceptibility 3) Determine herd risk factors for antimicrobial decreased susceptibility 4) Determine the mechanism of resistance for tetracycline.

The findings of the following material can be briefly summarized by addressing each objective above. Overall *Campylobacter* from both farm types was susceptible to most antimicrobials. Some resistance was demonstrated to ampicillin, kanamycin, tetracycline, sulfamethoxazole. The proportion of resistant isolates was only significantly higher for *Campylobacter* from conventional farms for tetracycline. Individual animal risk factors primarily include animal type. Calves were significantly at greater odds for decreased susceptibility for kanamycin, tetracycline and ampicillin. Some animal treatments were associated with increased odds of decreased susceptibility. Farm management risk factors that were associated with decreased risk include many of common sense hygiene, such as moving calf hutches in between calves, disinfecting milk buckets, and separating maternity areas from sick cows. The use of some antimicrobials was associated with decreased susceptibility. However, many of the patterns were not clear-cut and may include exposure to drugs other than the antimicrobial of interest in the outcome. It was confirmed that tetracycline resistance was conferred by the genetic determinant *Tet O*. Also several isolates became susceptible during the regrowth period, which supports plasmid carriage.

APPENDICES

APPENDIX A

Herd Recruitment Letter

May 1, 2000

Dear Dairy Producer,

Michigan State University's College of Veterinary Medicine would like to recruit dairy farms in Michigan for a research study we will be conducting in association with the USDA. The purpose of the study is to evaluate food safety issues in the dairy industry. This is an "observational study" which means we will first summarize a number of management practices and then compare what we find by collecting certain samples from animals and the environment.

Across the Midwest and Northeast, a total of 98 conventional and 32 organic dairy farms will be recruited to participate in Minnesota, Wisconsin, Michigan, and New York. Farms that are enrolled in the study will be visited every two months for a period of one year for the collection of samples. Sampling should begin in the summer of 2000, and the project should be completed in three years. There will also be a very small number of farms that will be sampled weekly for a period of three months for comparison purposes. We would like to be able to collect animal and environmental samples as well as associated records as efficiently as possible so as not to be inconvenient to the farmer or require much assistance.

Benefits for individual producers and the Michigan Dairy Industry:

The identity of farms participating in the study will remain anonymous and information obtained will not be used for regulatory purposes. Each farm will get results of the overall study and results about their individual farm. Participation in this study will allow producers to gain information relative to their animal health and general farm practices and compare them to the results of samples taken.

Local veterinarian involvement:

We will not be asking local veterinarians associated with herds enrolled in the study for assistance with sampling or data collection. Our researchers and sampling assistants will be gathering this information in a manner so as to inconvenience the dairy producers as little as possible. We also will not provide management advice regarding the results of the culturing information. Rather, we would like the local veterinarians to be involved in consulting and correcting management issues if our results indicate that there may be a problem with certain organisms (bacteria) or herd management practices.

How to get involved:

Please return the enclosed postcard if you are interested in participating in the project. Depending on responses, we will be using the information provided to enroll your herd into the appropriate category. *If you do not want to participate at this time, we request that you still return the postcard so that you can be removed from our future mailing.*

If you have question or comments, please contact:

Dr. Lisa W. Halbert at (517) 353-0847, email halbertl@cvm.msu.edu
or Dr. John B. Kaneene at (517) 353-5941, email kaneene@cvm.msu.edu

Sincerely,

Lisa W. Halbert, DVM
Graduate Research Assistant

APPENDIX B

Herd Enrollment Postcard

Dairy Food Safety Study

Your Name:_____
Phone Number: (_____)_____
Total Number of Cows (milking & dry):_____
Form of Records Used: ☐ DHIA ☐ Computerized
☐ Other, please specify:_____
Do animals have permanent ID? (e.g. eartag):_____
Do you raise calves < 2 months:_____
What percent of the herd is Holstein:_____
Do you ship milk all year:_____**or seasonally :**_____

Please check appropriate box.

☐ **Yes, I would be interested in participating in this study**

☐ **No, I would not be interested in this study.**
Please list reason: _____

._____.
._____.
._____.
._____.

Dr. John B. Kaneene
Dairy Food Safety Study
Population Medicine Center
A 109 Veterinary Medical Center
Michigan State University
East Lansing, MI 48824-1314

APPENDIX C

Initial Herd Questionnaire-

Conventional Dairy Version

Initial Questionnaire

Risk Factors for Salmonella and Campylobacter Infections and Drug Resistance in Dairy Cattle

This in-person questionnaire is to be given once for each producer e.g., at the initial herd visit. A much shorter questionnaire will be used to collect data that changes frequently.

Producer Information:

Farm name:_____

Owner(s) name:_____

Contact person or herdsman (if different from owner):_____

Farm Address:_____

Business Address (if different from above)_____

Home Phone:( )_____ Fax:( )_____

Barn Phone:( ) _____

E-mail:_____

Herd Veterinarian:_____

DHIA Number (if applicable):_____

Directions to farm:_____

_____

Person to whom survey is administered_____

Survey administrator_____

Date of next visit_____

A. Inventory—Herd Size

1. As of today, what is your inventory of the following groups of **dairy cattle**?

	Lactation 1*	Lactation 2 & up*	Total
A. Milking cows	i	ii	iii
B. Dry cows	iv	v	vi
C. Total cows (add totals of A. and B. above)			vii
D. Preweaned (milk-fed) heifer calves			viii
E. Weaned replacement calves and heifers**			ix
F. Other youngstock***			x
G. Bulls ****			xi
H. Total cattle (Add C-G above)			xii

* Lactation numbers here refer to the current lactation in the case of milking cows and to the lactation just completed for dry cows.

** "Weaned replacement calves and heifers" here means all female animals that will be kept as replacement cows, have not yet calved, and are no longer receiving milk or milk replacer as part of the diet.

*** "Other youngstock" here means **all animals that will not be kept as replacements** that are weaned or will be kept up to or past weaning (e.g., steers and heifers raised for beef—exclude calves that are only kept for a short period after birth)

**** Include only bulls kept for breeding purposes (e.g., breeding age bulls or younger bulls being saved for breeding purposes)

2. As of today, how many of the total **milk cows (both milking and dry)** were:

(NOTE: Add up the total cows in 1.C. and compare to 2.D. as a check before moving on to next page. These numbers should be the same—if not, investigate to see where the problem is)

A. Born and raised on this operation? (refers to all sites managed by this operation)..... _____head

B. Born here but raised elsewhere? (refers to contract rearing: in case they have done this in past but are not now) ..... _____head

C. Not born on this operation? ..... _____head

D. Total of A. + C. (Should equal 1.C. above.)..... _____head

3. This question refers to animals **other than dairy cattle** on this operation.

Within the last 12 months, have any of the following types of animals been present on this operation? If so, please indicate whether these animals had physical contact* with any of this operation's dairy cows or heifers, or their feed, minerals, or water supply.

	Present on operation?	Physical contact*?
A. Beef cattle?	☐ Yes ☐ No	☐ Yes ☐ No
B. Chickens, turkeys, domestic geese, or other poultry?	☐ Yes ☐ No	☐ Yes ☐ No
C. Horses or other equines (such as ponies, donkeys, mules, burros, etc.)?	☐ Yes ☐ No	☐ Yes ☐ No
D. Pigs?	☐ Yes ☐ No	☐ Yes ☐ No
E. Sheep?	☐ Yes ☐ No	☐ Yes ☐ No
F. Goats?	☐ Yes ☐ No	☐ Yes ☐ No
G. Farmed (confined to a pen) exotic animals (such as deer, llamas, ostriches, etc.)? Specify:_____	☐ Yes ☐ No	☐ Yes ☐ No
H. Dogs?	☐ Yes ☐ No	☐ Yes ☐ No
I. Cats?	☐ Yes ☐ No	☐ Yes ☐ No
J. Wild geese?	☐ Yes ☐ No	☐ Yes ☐ No
K. Other animals? _____	☐ Yes ☐ No	☐ Yes ☐ No

* As used here, "physical contact" means nose-to-nose contact or sniffing/touching/licking each other, including through a fence.

B. Herd Expansion Status

4. Were any of the following groups of animals brought onto this operation from outside sources **during the last 12 months**?

	Brought onto operation?	IF YES, How many?	IF YES, How many of these animals were isolated* upon arrival?	IF YES, On average, how long were they isolated* (in days)?
A. Prewean calves?	☐ Yes ☐ No			Days
B. Weaned dairy	☐ Yes ☐ No			Days
C. Dairy cows?	☐ Yes ☐ No			Days
D. Bulls?	☐ Yes ☐ No			Days
E. Other cattle,	☐ Yes ☐ No			Days
E. Total.				

* "Isolated" here means that the animal(s) is held for a period of time in a separate pen or other facility where nose-to-nose contact with cattle in the existing herd is prevented.

5. In the last 12 months, what is the largest number of dairy cows or weaned heifers that were introduced to the herd from outside sources **within a period of one week**. ..... _____head

C. Housing

6. Which one of the following types of milking facilities did this operation primarily use during the past 12 months? (Circle the appropriate letter A-D)

   A. Pit parlor?

B. Flat parlor or step-up milking facility?

C. Tie Stall or stanchion barn milking facilities?

D. Any other type of milking facility?
(specify)_____

7. What housing facilities did this operation use during the past 12 months for the following (check all that apply):

	Hutch	Freestall	Tie Stall	Calf is tied in stanchion or tie stall barn	Ind. animal area**	Multiple animal area***
A. Preweaned calves?						
B. Weaned heifers?						
C. Lactating dairy						
D. Maternity housing*?						

* "Maternity housing" here refers to where cows normally calve.

** "Individual animal area" here refers to a pen housing only one animal (e.g., individual calf pen) that is not covered by one of the previous options (e.g., if "hutch" has been selected, do not also mark "individual animal area" to refer to hutches).

*** "Multiple animal area" here refers to a pen housing multiple animal (including "super hutches") that is not covered by one of the previous options (e.g., if "freestall" has been selected, do not also mark "multiple animal area" to refer to freestalls).

8. During the past 12 months, approximately how many months of **daily access** to outside areas did the following groups of dairy animals have? (Enter "0" if no access)

	Drylot	Pasture	
		Does not provide at least 90% of roughage in ration)	Provides ≥ 90% of roughage in ration
A. Weaned dairy heifers?	Months	months	months
B. Lactating dairy cows?	Months	months	months

C. Dry cows?	Months	months	months
D. Maternity, close-up, or recently fresh cow housing?	Months	months	months

9. Is maternity housing* in a separate pen or facility from other lactating cows? ..... ☐ Yes ☐ No

* "Maternity housing" here refers to where cows normally calve.

10. Which of the following bedding types are typically used for the following groups of animals? Mark bedding types for each group of cattle using letters A-F corresponding to how often the bedding is changed. (e.g., if inorganic bedding for lactating cows is changed monthly, but organic bedding for lactating cows is changed every 2-3 days, put "B" in "other organic bedding" column and "E" in "inorganic bedding" column for lactating cows.)

	For each bedding type, put a letter A-F (select from list below) corresponding to how often the bedding is changed or added to		
	Dried manure	Other organic bedding*	Inorganic bedding**
Lactating cows			
Maternity, close-up, or recently fresh cows			
Sick cows			
Preweaned (milk-fed) calves			

A. Daily.
B. Every 2-3 days.
C. Weekly (more than 3 days, less than 8 days)
D. 2-3 times per month
E. Monthly
F. Greater than monthly

* "Organic bedding" here includes any organic materials used for bedding, such as straw, sawdust, newspaper, corn cobs or stalks, excluding dried manure.

** "Inorganic bedding" here includes any inorganic materials such as sand, rubber tires or mats, mattresses, crushed limestone, etc.

D. Feed and Water System

11. Do you feed a total mixed ration (TMR) to lactating dairy cows? ..... YES ☐ ☐ No

12. In the last 60 days, which of the following feeds have been used in the following groups of dairy animals? Include only **purchased feeds or feeds obtained from off-farm sources**. Check all that apply

Type of Feed	High-Producing Cows *	Other Milking Cows*	Dry Cows
A. Whole cottonseed/hulls			
B. Cottonseed meal			
C. Whole soybeans or soybean meal			
D. Bakery by-products			
E. Brewers by-products (includes distillers' grains)			
F. Blood meal			
G. Meat & bone meal (e.g., porcine-only or equine-only)			
G. Milk products (e.g., whey)			
H. Tallow/animal fat			
I. Other protein meal (e.g., meal from fish or poultry) Pleasespecify_____ _____			

* If high-producing cows are not fed differently from other cows, put N/A in "Other Milking Cows" column.

13. The following questions refer to the storage areas used for protein and concentrates fed to dairy cattle.

	Is storage area for this feed type in an enclosed building or other enclosed structure?	Does storage area for this feed type provide protection against moisture?	Does storage area provide protection Against birds or rodents?
A. Protein feeds	Yes☐ No☐	Yes☐ No☐	Yes☐ No☐
B. Concentrate	Yes☐ No☐	Yes☐ No☐	Yes☐ No☐

14. Which of the following coccidiostats or ionophores, if any, do you normally use for the following groups of animals? Include products used in feed, water, or milk replacer.

	Preweaned (milk-fed) calves	Weaned calves up to breeding	Heifers after breeding
Deccox (or other decoquinate product)			
Rumensin (or other monensin product)			
Bovatec (or other lasalocid product)			
Corid (or other amprolium product)			
Sulfaquinoxaline (many oral products)			
Other (Please specify)_____			

15. During the last 12 months, did cows drink from the following (check all that apply):

	Milk cows	Dry cows	Frequency **cleaned*** (times per year)	Frequency **disinfected**** (times per year)	List disinfectant
A. Automatic waterer—for individual cows (each has own cup or one cup shared by two cows)			_____Times/year	_____Times/year	
B. Automatic waterer—cows drink individually, but waterer shared by group			_____Times/year	_____Times/year	
C. Water tank—multiple cows can drink at once			_____Times/year	_____Times/year	
D. Lake, pond, stream, river, etc.—occasional use only					
E. Lake, pond, stream, river, etc.—seasonal main source (e.g., if primary source of water in summer is lake, pond, river, etc)					
F. Other: Please specify_____ _____			_____Times/year	_____Times/year	

* "Cleaned" here refers to removal of water from waterer and removal of scum or feed accumulation—regardless of whether a disinfectant is used.

** "Disinfected" means that after cleaning, a chemical disinfectant is used to sanitize waterer.

16. Is the water that dairy cattle drink usually chlorinated? Yes ☐ No ☐

17. What is the source of drinking water for cows? (Check all that apply)

A. Well

B. Municipal water

C. Surface water (stream, lake, spring, etc.)

D. Other (Please specify)_____

18. Is the ration for close-up dry cows different from the ration for far-off dry cows (i.e., does this operation have a transition/close up ration)?..... Yes ☐ No ☐

19. Does this operation normally feed anionic salts in transition cow diets (e.g., during the last 2 to 3 weeks of gestation) Common anionic salts are the sulfates or chlorides of magnesium, calcium, or ammonium?. Yes ☐ No ☐

E. Calf Management and Feeding

20. Which **one** of the following methods **is used most frequently** for the first feeding of colostrum to newborn dairy heifer calves? (Colostrum is the first milk produced after a calf is born.) (Circle the appropriate letter A-D)

A. Calf is left with cow to nurse for a period of time (e.g., for 2-4 hours)

B. Hand feeding from bucket or bottle

C. Hand feeding using esophageal feeder

D. Do not get colostrum

Answer #21 only if B or C is circled.

21. How much colostrum is normally fed during the first 24 hours? (A calf bottle is typically 2 quarts) (Circle the appropriate letter A-C)

A. Two quarts or less

B. More than 2, but less than 4 quarts

C. Four quarts or more

22. During the past 60 days, what types of **milk** have **usually** been fed to preweaned calves **that are kept up to weaning**, after they have received colostrum? Do not include calves (e.g. bulls) that are kept for only a few days, and do not include diets that are not fed as a usual practice (e.g., if waste milk is always fed to calves whenever available, mark "yes" for "B" regardless of the number of times it was fed in the past two months. On the other hand, if waste milk was discarded more often than it was fed, mark "no" for "B").

	Included in diet? (Check all that apply)	If A or B is YES, Is the milk pasteurized?
A. Whole milk from untreated* cows	Yes ☐ No ☐	Yes ☐ No ☐
B. Whole milk from treated* cows (waste milk)	Yes ☐ No ☐	Yes ☐ No ☐
C. Milk replacer without antibiotics	Yes ☐ No ☐	
D. Milk replacer containing antibiotics	Yes ☐ No ☐	
E. Calf starter without antibiotics	Yes ☐ No ☐	
F. Calf starter containing antibiotics	Yes ☐ No ☐	
G. Other (specify)_____	Yes ☐ No ☐	

* "Treated cows" refers to cows that have been given antibiotics and are still within the milk withholding period. (A cow given Naxcel/Excenel is **not** considered a "treated cow" here).

Answer question #23 only if D. or F. is YES,

23. List the types of antibiotics used below. If unknown, ask to look at tag of bag/container. Include only antibiotics here.

_____

_____

24. How often is maternity housing used as a hospital area for sick* cows? (Circle appropriate letter A-C)

    A. More than once a month

    B. Less than once a month

    C. Never

* "Sick" as used here refers to cattle designated as sick by personnel on your farm or by a veterinarian. Include all illnesses that would result in cattle being segregated (e.g., placed in sick pen) and/or treated with systemic antibiotics. This would include, but is not limited to lameness, respiratory disorders, and diarrhea.

25. After removal from the dam, at what age do heifers first have direct contact with adult cows in the herd? _____months

26. Which of the following best represents your normal practice regarding the cleaning of calf **milk** buckets or containers between feedings? (Circle the appropriate letter A-C)

    A. Between each feeding, all calf milk buckets or containers **washed with water only**.

    B. Between each feeding, all calf milk buckets or containers **washed and disinfected** . → List

    C. Buckets or containers not washed or disinfected between feedings on a routine basis.

27. Are preweaned (milk-fed) calves fed milk or calf starter on an individual basis (e.g., individual bucket in hutch or individual calf pen, as opposed to group feeding where a common trough is used)? ..... Yes ☐ No ☐

28. Are individual calf pens or hutches washed and/or disinfected on a regular basis? (Circle the appropriate letter A-D.)

A. Washed with water only. _____times per year

B. Washed and disinfected. _____times per year List disinfectant_____

C. Not washed or disinfected.

D. Calf pen or hutch is not used.

29. How often are individual hutches moved to a new location? (Choose the appropriate letter A-D)

A. Every time a calf is weaned. (Before introducing each new calf.)

B. Not after every weaning, but on a regular basis _____times per year

C. Calf hutches are not relocated.

D. Calf hutches are not used.

30. Do personnel on your farm use any of the following precautionary practices when handling calves? (Check all that apply)

	After handling each calf	When finished with all calves (e.g., before entering a different area of the farm)	Do not routinely use this practice when handling calves
A. Wash boots or use boot dip			
B. Wash hands after handling calf or use disposable gloves			

31. Is unpasteurized milk that is produced on this operation consumed by family members, farm workers, or others?

A. Unpasteurized milk from this operation is consumed.

B. Home pasteurizer is used for milk produced on this operation.

C. Unpasteurized milk is not consumed. All milk consumed is purchased.

G. Production and Health

32. During the last six months, which of the following best describes the average bulk tank somatic cell count for milk shipped? (Circle the appropriate letter A-F below)

A. <100,000
B. 100,000-199,000
C. 200,000-299,000
D. 300,000-399,000
E. 400,000-499,000
F. 500,000+

33. During the last six months, which of the following best describes the average bacterial count (aka: standard plate count, plate loop count) for milk shipped? (Circle the appropriate letter A-E)

Colony forming units per millimeter (cfu/ml)

A. 0-24,999
B. 25,000-49,999
C. 50,000-74,999
D. 75,000-99,999
E. 100,000+

34. Do you use DHIA or other computerized records? ☐ Yes ☐ No

If YES, answer

If NO, go to #36

35. What is your current rolling herd average for milk production? ..... _____Annual

36. What is your **average** pounds of milk produced per day? (This question is to be asked for purposes of approximating a rolling herd average if one is not available by DHIA or other records. ..... _____

37. Are sick* cattle placed in a pen or facility separate from lactating cows? ..... Yes ☐ No ☐

* "Sick" as used here refers to cattle designated as sick by personnel on your farm or by a veterinarian. Include all illnesses that would result in cattle being

segregated (e.g., placed in sick pen), and/or treated with systemic antibiotics. This would include, but is not limited to lameness, respiratory disorders, and diarrhea.

38. Within the past two years, have any of your dairy cattle been positively diagnosed (i.e., by evidence of positive fecal culture or other laboratory test) with any of the following diseases? (Circle all that apply)

    A. Salmonella

    B. Johne's disease

    C. Bovine Viral Diarrhea (BVD)

    D. No cattle have been diagnosed with any of the diseases above.

39. Do you normally vaccinate cows with any of the following vaccines? (Circle all that apply)

    A. J5 (Enviracor by Upjohn or J. Vac J5 by Rhone Merieux)

    B. Endovac Bovi

    C. Salmonella bacterin vaccine

40. Within the last 60 days, how many dairy cattle within the following groups had diarrhea or died?

	Number of animals with diarrhea lasting at least 24 hours?	Number of deaths among animals with diarrhea lasting at least 24 hours	Number of total animals that have died
Preweaned calves			
Weaned heifers			
Milk cows (milking or dry)			

41. Are any of the following methods of rodent control routinely used on this operation? (Circle all letters A-D that apply.)

A. Chemicals/bait?

B. Traps?

C. Cats?

D. Other methods? (specify)_____

H. Manure Management

42. Do you use any of the following to remove manure from cow housing areas? (Circle all letters A-E that apply)

A. Gutter cleaner

B. Tractor (bucket loader or skid steer)

C. Hand fork or shovel

D. Alley scraper--mechanical

E. Alley flushed with water If so, is the water recycled? ☐ Yes ☐ No

F. Other (specify)_____

43. Are any of the following waste **storage** systems used on this operation? (Circle all letters A-K that apply)

A. Below floor or deep pit

B. Anaerobic lagoon with cover

C. Slurry storage in earth-basin

D. Anaerobic lagoon without cover

E. Slurry storage in Slurrystore® (or similar storage structure)

F. Aerated lagoon

G. Manure pack (inside barn)

H. Outside storage within dry lot or pens

I. Outside storage for solid manure not in dry lot or pen

J. Storage of solid manure in a building without cattle access

K. Other storage system used or no storage system used (specify)_____

44. You may respond to this question in miles or feet. What is the distance between the manure storage area and the nearest:

A. Well? _____miles..... or_____feet

B. Waterway or body of water? _____miles..... or_____feet

45. Which of the following methods are used to dispose of manure on owned or rented land? (Circle all letters A-E that apply)

A. Irrigation
B. Slurry (surface application)
C. Broadcast/solid spreader
D. Slurry (subsurface application)
E. Other method (specify)_____
F. Do not apply manure on owned or rented land.

46. In this question, the term "roughage" means hay, fresh chop forage, or pasture that dairy animals may eat or graze. Do cows eat or graze on roughage obtained from fields where manure in solid or liquid form was applied to the surface but not plowed under during the same growing season?..... ☐ Yes ☐ No

If YES, answer #47

47. How many days do you wait after applying manure to a field before cows are allowed to eat or graze the roughage from that field?_____days

48. Do you use a loader bucket on a tractor or skid steer to move feed?..... ☐ Yes ☐ No

If NO, go to Section I

If YES, answer #49

49. Do you use separate loader buckets for moving feed and for handling manure? (Circle the appropriate letter A-C)

A. Yes, use separate buckets.

B. No, do not use separate buckets.

C. Do not use this equipment for handling manure.

If B. is circled, answer

50. After you have used the loader bucket for handling manure, do you do any of the following before using it for feed?: (Circle the appropriate letter A-D)?

A. Rinse bucket with water only.

B. Power wash bucket with high pressure water.

C. Wash and disinfect bucket. List disinfectant_____

D. Do not wash or disinfect bucket

I. Antimicrobial Use

51. Which of the following best describes the use of dry cow tubes (intramammary infusions) used to treat your cows at final milk out? (Circle one of the following letters A-C)

A. Dry treat all 4 quarters on all or almost all the cows

B. Dry treat selected cows only, 1 or more quarters

C. Do not dry treat any cows

52. Does this operation routinely record antibiotic treatment for the following groups of cattle in some way?

		If YES, what types of records are kept? (Check all that apply)			
	Antibiotic treatment recorded?	Comput-ized	Barn sheet, log, or notebook	Calendar	Other specify
A. Lactating cows	☐ Yes ☐ No				
B. Non-Lact Cows	☐ Yes ☐ No				
C. Calves and heifers	☐ Yes ☐ No				

53. Where do you get recommendations on the following aspects of antibiotic use? (Check all that apply)

	Vet	Pharm. Rep	Personal Exper	Product label	Other farmers	Other- Please specify
Recommeded use						
Dosage						
Withdrawal Time						

54. When you treat **respiratory disease in adult cows** with antibiotics, what antibiotics do you normally use? (Circle all that apply)

A. Naxcel/Excenel (ceftiofur)

B. Tetracyclines (e.g., Liquamycin--LA-200)

C. Penicillin

D. Ampicillin (e.g., Polyflex)

E. Albon (sulfadimethoxine)

F. Others (please specify)_____

55. When you treat **respiratory disease in calves and heifers** with antibiotics, what antibiotics do you normally use? (Circle all that apply)

A. Naxcel/Excenel (ceftiofur)

B. Nuflor (florfenicol)

C. Penicillin

D. Tetracyclines (e.g., Liquamycin--LA-200, Oxy-Tet-100)

E. Ampicillin (e.g., Polyflex)

F. Micotil (tilmicosin)

G. Others (please specify)_____

56. When you treat **calf scours** with systemic antibiotics, what antibiotics do you normally use (oral or injectable)? (Circle all that apply)

A. Panmycin boluses (tetracycline) B. ..... Spectam (spectinomycin)

C. Nuflor (florfenicol) D. Trimethoprim-Sulfa

E. Others (please specify)_____

F. Do not use systemic antibiotics for calf scours.

57. When you treat **mastitis** with systemic (oral or injectable) antibiotics, what antibiotics do you normally use? Do not include intramammary antibiotics. (Circle all that apply)

A. Polyflex (ampicillin)
B. Amoxi-Inject (amoxicillin)
C. Penicillin
D. Erythromycin (e.g., Gallimycin)
E. Others (please specify)_____
F. Do not use systemic antibiotics for mastitis.

58. When you treat **metritis or retained placenta (RP)** with systemic (oral or injectable) antibiotics, what antibiotics do you normally use? (Circle all that apply)

A. Naxcel/Excenel
B. Penicillin
C. Ampicillin (e.g., Polyflex)
D. Others (please specify)_____
E. Do not use systemic antibiotics for metritis/retained placenta.

59. When you treat **foot problems in adult cows** with systemic antibiotics (oral or injectable), what antibiotics do you normally use Do not include topical treatments such as in foot wraps. (Circle all that apply)

A. Ampicillin (e.g., Polyflex)
B. Penicillin
C. Albon (sulfadimethoxine)
D. Naxcel/Excenel (ceftiofur)
E. Tetracyclines (e.g., Liquamycin--LA-200)
F. Ampicillin (Polyflex)
G. Others (please specify)_____
H. Do not use systemic antibiotics for foot problems.

60. Do you routinely use **antibiotics in footbaths** to control or treat lameness? ..... ☐ Yes ☐ No

A. **If YES**, do you use the antibiotics in footbaths on a continuous basis (i.e., all year long)? ..... ☐ Yes ☐ No

B. Please list what antibiotics are used, if any:_____

_____

_____

61. Do you routinely **use any medications in feed or water in weaned calves or heifers** (other than coccidiostats)? ☐ Yes ☐ No

A. **If YES**, do you use the additives on a continuous basis? ☐ Yes ☐ No

B. Please list what feed or water additives are used, if any:_____

62. Approximately what percent of the following groups of cattle have received at least one antibiotic injection (or oral dose of antibiotics) within the past two months? Include treatments given by personnel on your farm or by your veterinarian. Do not include intramammary or topical administration of antibiotics. (Make only **one check per column**)

	Milk cows (milking or dry)	Bred heifers	Heifer calves (weaned or preweaned)
0 %			
1-10 %			
11-25 %			
26-50 %			
51-75 %			
76-100 %			

63. Within the past two months, approximately how much of the following antibiotics have you used? **Fill in only one column per row in the table below.**

	Approximate number of bottles used, including bottle size (put "0" if do not use or if used less than one bottle in past two months)	Approximate number of doses*, if less than one bottle was used.
Penicillin-type Includes penicillin, amoxicillin (Amoxi-inject), ampicillin (Polyflex)	_____bottles of size_____ml or g	_____doses
Cephalosporin-type Includes ceftiofur (Naxcel, Excenel)	_____bottles of size_____ml or g	_____doses
Tetracycline-type (includes LA-200, Oxy-Tet-100)	_____bottles of size_____ml or g	_____doses
Sulfonamides Includes sulfadimethoxine (Albon)	_____bottles of size_____ml or g	_____doses

Florfenicol (NuFlor)	_____bottles of size_____ml or g	_____doses
Other antibiotics Includes tilmicosin (Micotil), Erythromycin (Gallimycin), and any others not covered in the groups above.	_____bottles of size_____ml or g	_____doses

* A "dose" here means one administration of antibiotic. E.g., if you give 20 ml of Naxcel to a cow, that is one dose. If you give another 20 ml the next day to the same cow, that is another dose.

Glossary of Terms

The terms listed below are defined according to how they are meant to be used in this survey.

Calving Interval: the time from one calving to the next calving

Colostrum: The first milk produced after a calf is born

Heifer: Non-lactating weaned female animal that has not yet calved.

Inorganic bedding includes any inorganic materials such as sand, rubber tires or mats, mattresses, crushed limestone, etc.

Isolated/Isolation: A newly acquired animal(s) is held for a period of time in a separate pen or other facility where nose-to-nose contact with cattle in the existing herd is prevented

Maternity housing refers to where cows normally calve.

Organic bedding includes any organic materials used for bedding, such as straw, wood products such as sawdust or newspaper, corn cobs or stalks, excluding dried manure.

Physical Contact: means nose-to-nose contact or sniffing/touching/licking each other, including through a fence

Sick as used here refers to cattle designated as sick by personnel on your farm or by a veterinarian. Include all illnesses that would result in cattle being segregated, and/or treated with systemic antibiotics. This would include, but is not limited to lameness, respiratory disorders, and diarrhea.

Treated cows means cows that have been given antibiotics.

Youngstock: means all animals that are past weaning age and will not be kept as replacements (e.g., steers and heifers raised for beef)

APPENIDIX D

Initial Herd Questionnaire-

Additional Organic Dairy Questions

A. After **dairy cattle** on this operation are treated with antibiotics, are they normally separated from the rest of the organic herd (i.e., sold or moved to a location with no physical contact with the rest of the organic herd)?

☐ Yes ☐ No ☐ Antibiotics are not used

If YES, answer #53

Go to #54

B. Is the separation permanent? (i.e., animal is sold or remains physically isolated from the rest of the organic herd?

☐ Yes

☐ No → Please specify length of separation_____days

C. After **dairy cows or heifers** on this operation are treated with antibiotics are they later used for organic milk production after a withdrawal period has passed?

☐ Yes Please specify length of withdrawal period_____days

☐ No

☐ Antibiotics are not used.

D. After **dairy cattle** on this operation are treated with antibiotics are they later used for organic meat production after a withdrawal period has passed?

☐ Yes Please specify length of withdrawal period_____days

☐ No

☐ Antibiotics are not used.

E. What treatments or therapy do you normally use to treat **respiratory disease in adult cows?**

_____

_____

F. Do you or have you used antibiotics to treat **respiratory disease in adult cows?** ..... ☐ Yes No ☐

IF YES, ask:

1) When you treat **respiratory disease in adult cows** with antibiotics, what antibiotics do you normally use? (Circle all that apply)

a. Naxcel/Excenel (ceftiofur)
b. Tetracyclines (e.g., Liquamycin--LA-200)
c. Penicillin
d. Ampicillin (e.g., Polyflex)
e. Albon (sulfadimethoxine)
f. Others (please specify)_____

G. What treatments or therapy do you normally use to treat **respiratory disease in calves and heifers?**

_____

H. Do you or have you used antibiotics to treat **respiratory disease in calves and heifers?** ..... ☐ Yes ☐ No

IF YES, ask:

2) When you treat **respiratory disease in calves and heifers** with antibiotics, what antibiotics do you normally use? (Circle all that apply)

a. Naxcel/Excenel (ceftiofur)
b. Nuflor (florfenicol)
c. Penicillin
d. Tetracyclines (e.g., Liquamycin--LA-200)
e. Ampicillin (e.g., Polyflex)
e. Micotil (tilmicosin)
f. Others (please specify)_____

I. What treatments or therapy do you normally use to treat **calf scours?**

_____

J. Do you or have you used antibiotics to treat **calf scours**? ..... ☐ Yes ☐ No

IF YES, ask:

When you treat **calf scours** with antibiotics, what antibiotics do you normally use? (Circle all that apply)

3)

a. Panmycin boluses (tetracycline)
b. Spectam (spectinomycin)
c. Nuflor (florfenicol)
d. Trimethoprim-Sulfa
e. Others (please specify)_____

K. When you use a systemic treatment for **mastitis** (i.e., not intramammary or topical), what do you normally use? If no systemic treatments are used for mastitis, put N/A below.

_____

L. Do you or have you used antibiotics for systemic treatment (e.g., oral or injectable) of **mastitis**?..... ☐ Yes ☐ No

IF YES, ask:

When you treat **mastitis** with systemic antibiotics, what antibiotics do you normally use? (Circle all that apply)

4)

a. Polyflex (ampicillin)
b. Amoxi-Inject (amoxicillin)
c. Penicillin
d. Erythromycin (e.g., Gallimycin)
e. Others (please specify)_____

M. When you use a systemic treatment for **metritis or retained placenta (RP)** (i.e., not topical or intrauterine), what do you normally use? If no systemic treatments are used for metritis or retained placenta, put N/A below.

_____

N. Do you or have you used antibiotics for systemic treatment (e.g., oral or injectable) of **metritis or retained placenta (RP)**?.....5) ☐ Yes ☐ No

IF YES, ask:

When you treat **metritis or retained placenta (RP)** with systemic antibiotics, what antibiotics do you normally use? (Circle all that apply)

a. Naxcel/Excenel
b. Penicillin
c. Ampicillin (e.g., Polyflex)
d. Others (please specify)_____

O. When you use a systemic treatment for **foot problems in adult cows** (i.e., not topical), what do you normally use? ? If no systemic treatments are used for foot problems in adult cows, put N/A below.

_____

P. Do you or have you used antibiotics for systemic treatment (e.g., oral or injectable) of **foot problems in adult cows)**? ..... ☐ Yes ☐ No

IF YES, ask:

When you treat **foot problems in adult cows** with systemic antibiotics, what antibiotics do you normally use? (Circle all that apply)

6)

a. Ampicillin (e.g., Polyflex)
b. Penicillin
c. Albon (sulfadimethoxine)
d. Naxcel/Excenel (ceftiofur)
e. Tetracyclines (e.g., Liquamycin--LA-200)
f. Ampicillin (Polyflex)
g. Others (please specify)_____

Q. Do you routinely use **treated footbaths** to control or treat lameness? ..... ☐ Yes ☐ No

A. **If YES**, do you use the footbaths on a continuous basis (i.e., all year long)?..... ☐ Yes ☐ No

B. Please list what is used in the footbaths:_____

_____

_____

R. Do you routinely **use any medications in feed or water in weaned calves or heifers?** ..... ☐ Yes ☐ No

A. **If YES**, do you use the additives on a continuous basis? ..... ☐ Yes ☐ No

B. Please list what feed or water additives are used, if any:_____

_____

_____

APPENDIX E

Herd Visit Questionnaire

Herd Visit Questionnaire

Risk Factors for Salmonella and Campylobacter Infections and Drug Resistance in Dairy Cattle

This short questionnaire is to be given every two months (at each sampling visit) in order to capture management and inventory changes that may have occurred since the initial questionnaire was given.

IMPORTANT: Note that on questions 4 and 5, the questionnaire administrator should pencil in answers from the last administration of the questionnaire and note any changes between previous answers and what is being fed today. Ask questions in the format "Are you still feeding blood meal to high-producing cows?" for feeds that were previously fed. For feeds that weren't fed in the past, make sure they are not now feeding them, such as by asking "Are you feeding any blood meal to any cows now?" and, if so, ask further which groups are being fed blood meal.

Date:_____

Study ID number:_____

Person to whom herd visit questionnaire is administered_____

Herd visit questionnaire administrator_____

Date of next visit_____

S. As of today, what is your inventory of the following groups of **dairy cattle**?

	Total
A. Total cows (milking and dry)	7)
B. Preweaned (milk-fed) heifer calves	8)
C. Weaned replacement calves and heifers*	9)

* "Weaned replacement calves and heifers" here means all female animals that will be kept as replacement cows, have not yet calved, and are no longer receiving milk or milk replacer as part of the diet.

T. Were any of the following groups of animals brought onto this operation from outside sources **during the last 60 days**?

	Brought onto operation? 1 = YES 2 = NO	IF YES, How many were brought onto operation?
A. Preweaned (milk-fed) calves?	10) ☐ Yes ☐ No	11)
B. Weaned dairy calves or	12) ☐ Yes ☐	13)
C. Dairy cows?	14) ☐ Yes ☐ No	15)
D. Bulls?	16) ☐ Yes ☐ No	17)
E. Other cattle, including beef?	18) ☐ Yes ☐ No	19)
E. Total.		20)

* "Isolated" here means that the animal(s) is held for a period of time in a separate pen or other facility where nose-to-nose contact with cattle in the existing herd is prevented.

U. WitHin the last 60 days, how many dairy cattle within the following groups had diarrhea or died?

Coding instructions : 1 = checked; 2 = unchecked	Number of animals with diarrhea lasting at least 24 hours?	Number of deaths among animals with diarrhea lasting at least 24 hours	Number of total animals that have died
Preweaned calves	21)	22)	23)
Weaned heifers	24)	25)	26)
Milk cows (milking or dry)	27)	28)	29)

V. Have the ration ingredients for milking and dry cows changed since the last time our questionnaire was given? Compare answers from the previous questionnaire with what is now being fed and note any changes in the table below. Include only **purchased feeds or feeds obtained from off-farm sources**. (Check all that apply).

Type of Feed	High-Producing Cows*	Other Milking Cows*	Dry Cows
A. Whole cottonseed/hulls	30)	31)	32)
B. Cottonseed meal	33)	34)	35)
C. Whole soybeans or soybean meal	36)	37)	38)
D. Bakery by-products	39)	40)	41)
E. Brewers by-products (includes distillers' grains)	42)	43)	44)
F. Blood meal	45)	46)	47)
G. Meat & bone meal (e.g., porcine-only or equine-only)	48)	49)	50)
G. Milk products (e.g., whey)	51)	52)	53)
H. Tallow/animal fat	54)	55)	56)
I. Other protein meal (e.g., meal from fish or poultry) Please specify_____ 57)	58)	59)	60)

* If high-producing cows are not fed differently from other milking cows, put **N/A** in the "Other Milking Cows" column.

W. Have the types of **milk or calf starter** fed to preweaned calves changed since the last time our questionnaire was given? Compare answers from the previous questionnaire with what is now being fed and note any changes in the table below. Include only calves **that are kept up to weaning**, after they have received colostrum.

	Included in diet?
A. Whole milk from untreated* cows	61) ☐ Yes ☐ No
B. Whole milk from treated* cows (waste milk)	62) ☐ Yes ☐ No
C. Milk replacer without antibiotics	63) ☐ Yes ☐ No
D. Milk replacer containing antibiotics	64) ☐ Yes ☐ No
E. Calf starter without antibiotics	65) ☐ Yes ☐ No
F. Calf starter containing antibiotics	66) ☐ Yes ☐ No
G. Other (specify)_____ 67)	68) ☐ Yes ☐ No

Answer question #6 only if C, D, E, or F. is YES

* "Treated cows" refers to cows that have been given antibiotics and are still within the milk withholding period. (A cow given Naxcel/Excenel is **not** considered a "treated cow" here).

X. List the types of antibiotics used and the brand names of the milk replacer or calf starter below. If unknown, ask to look at tag of bag/container.

Antibiotics used, if any
_____ 69)

Brand name of milk replacer_____ 70)

Brand name of calf starter_____ 71)

1 = YES
2 = NO

Y. Within the past 60 days, have you **used any medications in feed or water in weaned calves or heifers** (other than coccidiostats)? ..... 72)..... Yes ☐ No ☐

A. **IF YES**, Please list the feed or water medications used. Include brand name of additive, medication name, and duration of use:

_____
_____73)

Z. Within the past 60 days, have you **used any medications in feed or water in adult cows?** .....74) ☐ Yes ☐ No

A.**If YES**, Please list the feed or water medications used. Include brand name of additive, medication name, and duration of use:

_____
_____75)

AA. Within the past 60 days, approximately how much of the following antibiotics have you used? **Fill in only one column per row in the table below.**

		Approximate number of bottles used, including bottle size (put "0" if do not use or if used less than one bottle in past two months) # bottles size of bottle (# ml or g) units (ml or g) coding: (1 = ml; 2 = g)	Approximate number of doses*, if less than one bottle was used.
Penicillin-type	Pencillin	94)_____ 95)_____ 96)_____	97) ___doses
	Amoxicillin (e.g., Amoxi-inject)	98)_____ 99)_____ 100)_____	101) ___doses
	Ampicillin (e.g., Polyflex)	102)_____ 103)_____ 104)	105) ___doses
Cephalosporin-type Includes ceftiofur (Naxcel, Excenel)		106)_____ 107)_____ 108)	109) ___doses
Tetracycline-type (includes LA-200, Oxy-Tet-100)		110)_____ 111)_____ 112)	113) ___doses
Sulfonamides	Albon or other sulfas	114)_____ 115)_____ 116)	117) ___doses
	Trimethoprim-sulfa type (e.g., Tribrissen, SMZ-TMP, Primor)	118)_____ 119)_____ 120)	121) ___doses
Florfenicol (NuFlor)		122)_____ 123)_____ 124)	125) ___doses

Tilmicosin (Micotil)	126)_____127)_____ 128) _____	129) ____doses
LS-50 (Spectinomycin/Lincomycin soluble powder)	130)_____131)_____ 132) _____	133) ____doses
Other antibiotics(e.g., Spectam, Gentocin, Erythromycin, etc. Please specify)		

* A "dose" here means one administration of antibiotic. e.g., if you give 20 ml of Naxcel to a cow, that is one dose. If you give another 20 ml the next day to the same cow, that is another dose

Glossary of Terms

The terms listed below are defined according to how they are meant to be used in this survey.

Dose: as used here means one administration of antibiotic. e.g., if you give 20 ml of Naxcel to a cow, that is one dose. If you give another 20 ml the next day to the same cow, that is another dose.

Heifer: Non-lactating weaned female animal that has not yet calved.

Isolated/Isolation: A newly acquired animal(s) is held for a period of time in a separate pen or other facility where nose-to-nose contact with cattle in the existing herd is prevented

Medications: as used here refers specifically to antibiotics—it does not refer to probiotics, anthelmintics and other non-antibiotic medications.

Physical Contact: means nose-to-nose contact or sniffing/touching/licking each other, including through a fence

Preweaned calves: as used here means calves that are still receiving milk or milk replacer.

Treated cows: means cows that have been given antibiotics and are still within the milk withholding period. (A cow given Naxcel/Excenel is **not** considered a "treated cow" here).

Weaned: refers to animals that are no longer receiving milk or milk replacer.

Weaned replacement calves and heifers: here means all female animals that will be kept as replacement cows, have not yet calved, and are no longer receiving milk or milk replacer as part of the diet

APPENDIX F

Data Collection Sheet for Sampling

Study ID:_____ Date:_____

Environmental Sample ID	**Description**	**Comments**
E -1	Feedbunk	
E-2	Calf hutches-pens (4 –pooled)	
E-3	Sick Pen (4 –pooled)	
E-4	Freshening pen (4 –pooled)	
E-5	Water tank or Cups	
E-6	Lagoon or Manure pack	
E-7	Bulk Tank Milk	
E-8	Milk Filter	
E -9	Bird Droppings	
E-10	Cull Cow Hide Swab	Cow ID:
E-11	Cull Cow Hide Swab	Cow ID:

Cow ID	Animal Type	FreshDay BirthDay	Lactation Number	Health Status	Treatment Code	Treatment Date

APPENDIX G

Animal Health and Treatment Codes

Animal Codes

1 = Calf < 2 months
2 = Healthy cow
3 = Cull Cow
4 = Pre-Fresh (close up) Cow
5 = Fresh cow
6 = Sick Cow

Health Codes

0 = Healthy
1 = Metritis /RP
2 = Mastitis
3 = Pneumonia/ Respiratory
4 = Ketotic
5 = DA (L or R)
6 = Lame
7 = Diarrhea/Scours
8 = Milk Fever
9 = Peritonitis
10 = Hardware

Treatment Codes

Systemic only (injectable or oral)
Not intramammary or topical

0 = No treatment
1 = Penicillin
2 = PolyFlex
3 = Naxcel
4 = Amoxi-inject
5 = Oxy Tet 100
6 = LA Tet 200
7 = Nuflor
8 = Micotil
9 = Gentacin
10 = Albon
11 = Baytril
12 = Lincocin
13 = Erythromycin
14 = Spectam
15= LS-50
16 = SMZ-TMP

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