Tracking, Quantifying, Phenotyping and Genotyping of *Campylobacter* in Cattle and Pigs across the Farm to Fork Continuum

Dissertation

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Abstract

Salmonella and *Campylobacter* cause a significant number of illnesses annually in the United States and around the world, most of which are food-related. The objectives of this dissertation were to compare estimates of *Salmonella* and *Campylobacter* concentrations using most probable number (MPN) method, direct dilution, and quantitative real-time PCR (QPCR) in fecal, hide, carcass, and meat samples and to determine the clonality of selected *Campylobacter coli* isolates from the same pigs collected at each of the five sample types based on phenotype. Cattle and swine can be asymptomatic carriers of both of these pathogens. We first compared estimates of *Salmonella* and *Campylobacter* concentrations using most probable number (MPN) method, direct dilution, and quantitative real-time PCR (QPCR) in spiked fecal, hide, carcass, and meat samples from cattle and swine. Different sample types were spiked with known concentrations of *Salmonella enterica*, *Campylobacter jejuni* and *Campylobacter coli*. The results of this study indicated that there was a strong positive correlation between the pre- and post-spiking samples for all the sample types tested, *Salmonella* MPN results indicated there was a strong positive correlation between pre- and post-spiking concentrations for the MPN results from the bovine, and porcine, fecal samples, *Campylobacter* direct dilution revealed only the bovine feces for *C. coli* was significant for a strong positive correlation between initial spiking concentrations and post spiking values, and *Campylobacter* MPN results indicated there was a strong

positive correlation between the pre and post spiked samples for carcass and ground beef samples.

We next investigated the association between the concentration of *Salmonella* and *Campylobacter* pre- and post-harvest in cattle. Samples were collected from each of 98 individually identified cattle during the peri-harvest and post-harvest periodincluding: on farm (fecal sample), post-stunning and exsanguination (hide sponge and pre-fecal sample (lairage), pre-chilling (carcass sponge) and final product (ground meat). *Salmonella* and *Campylobacter* were cultured and quantified at each stage by using the direct dilution and most probable number method. *Salmonella* was not isolated from any sample, and there were no associations between *Campylobacter* concentrations for any two sample types.

We further investigated the same potential association between the concentration of *Salmonella* and *Campylobacter* pre- and post-harvest in swine. In this study, *Salmonella* was isolated from only two samples, but again there was no association between the isolation of *Campylobacter* on meat and the isolation of *Campylobacter* at any peri-harvest stage.

Our next step was to compare the concentration of *Campylobacter* measured by QPCR compared to direct dilution or MPN from 100 swine (5 sample types) and 98 cattle (5 sample types). Samples for this study were obtained from the previous two studies. There was no observed association when QPCR results were compared to either direct dilution or MPN in cattle or pigs. These results suggest that quantitative culture (direct dilution and MPN) would be the most appropriate means of quantifying *Campylobacter*

in cattle and swine samples. This was based on the observation that there was no agreement between the two methods and each of the positive samples for MPN or direct dilution were PCR confirmed thus it appears that the QPCR was producing false negatives suggesting inadequate sensitivity

Finally, we investigated the clonal relatedness of *Campylobacter coli* from pigs on farm and at processing. We completed antimicrobial susceptibility testing and multi-locus sequence typing (MLST) of these 47 isolates. *Campylobacter* isolates from farm, lairage, hide and carcass showed similar phenotypes and belonged to the same clonal groups based on MLST. Five new Sequence Types were identified (ST-4083, ST-4084, ST-4085, ST-4086, ST-4087). Our results indicated a high diversity of *C. coli* within one farm, and that meat products were contaminated with the same STs as those recovered in earlier stages of the harvest process.

I dedicate this dissertation to my children. May they always remember that anything is possible when you work hard, rely on God and never give up.

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Fields of Study

Major Field: Veterinary Preventive Medicine

Specializations: Molecular Epidemiology, College and University Teaching

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Chapter 1 Literature Review

1.1 Introduction

Campylobacter jejuni was first successfully cultured from stool in 1968 with the collaborative work of a medical doctor (Butzler) and a veterinarian (Dekeyser) (Butzler. 2004). Since that time, *Campylobacter spp.* have been recognized as a major cause of human gastrointestinal illness around the world. A report created in 1997 estimated the total cost of food-borne campylobacteriosis to be between $0.8 billion to $5.6 billion annually in the United States alone (Buzby, et al. 1997). An estimated 2 million people in the US are affected by *Campylobacter* infections each year (Samuel, et al. 2004). The majority of people infected with *Campylobacter* experience clinical signs including bloody diarrhea, abdominal cramps, and fever that typically last about a week. Most *Campylobacter* infections are localized in the GI tract and self-limiting. The CDC reports that approximately 124 people die each year in the US due to *Campylobacter* infections. Campylobacteriosis has also been associated with Guillain-Barré syndrome, an autoimmune disorder where the immune system is triggered and attacks the nerves causing paralysis that can last several weeks. Approximately one in every one thousand cases of campylobacteriosis results in Guillain-Barré syndrome and 40% of Guillain-Barré syndrome cases have been associated with a *Campylobacter* infection (Friedman, et al. 2000). Infants and children under the age of five are the highest risk of infection with this pathogen.

Campylobacteriosis cases are usually sporadic, but outbreaks have been reported. Sporadic cases are usually associated with improper handling and cooking of raw poultry, pork and beef and cross contamination of surfaces. Outbreaks are typically result from the consumption of contaminated raw milk or contaminated water. Additional risk factors associated with C*ampylobacter* infectious include consumption of undercooked meats, and direct contact with pets and farm animals (Frost. 2001, Deming, et al. 1987, Kapperud, et al. 1992, Hopkins, et al. 1984, Oosterom, et al. 1984). Campylobacteriosis is typically a self-limiting disease but in invasive cases antibiotics can help to reduce the severity and duration of symptoms. The most common antibiotics used to treat Camplobacter infections are macrolides (erythromycin, clarithromycin, or azithromycin) or fluoroquinolones (ciprofloxacin, levofloxacin, gatifloxacin, or moxifloxacin).

An interesting observation about *Campylobacter* is that it was demonstrated that the infectious dose of *Campylobacter jejuni* in humans was as low as 500 cells (Robinson. 1981). The high cost of this organism coupled with the low infectious does demonstrates the need for quantitative studies to better understand this organism's presence and risk in cattle and pigs from farm to table. This dissertation aims to better bridge the gap in understanding of the prevalence, quantity, and molecular epidemiology of *Campylobacter* associated with cattle and swine across the farm to table continuum. The following will be a review of the current status of scientific knowledge that has already been gained from this challenging organism.

1.2 *Campylobacter* isolation and identification

One of the challenges when working with *Campylobacter* is the lack of consistency between studies in the method of culturing the organism. C*ampylobacter* can also be a very challenging organism to grow in the laboratory, due to the thermophilic and microareophilic needs of *Campylobacter* sp of foodborne concern. There have been many different scientists who have developed methods that have allowed the successful culturing of *Campylobacter* sp. from various sample types (Bolton, et al. 1984, Hutchinson, et al. 1984, Lauwers, et al. 1978). There are similar objectives for the different steps of *Campylobacter* culture from enrichment, selection and identification. The enrichment step aims to provide enrichment for *Campylobacter* and limit the growth of other bacteria. The most common ingredients in the enrichment step include; peptones, glucose and yeast extract (for nutrients), ferrous sulphate, sodium metabisulphate and sodium pyruvate (to dissipate toxic compounds and to increase the aerotolerance of the organisms), lysed horse blood (oxygen quenching and to neutralize trimethoprim inhibitors), cycloheximide or amphotericin B (antifungal) and anitbiotics (most commonly vancomycin, cefoperazone, trimethoprim) then a 48 incubation at 42°C under microaerophilic conditions (O_2 5-15% and CO_2 5-12%). The selection step involves the same ingredient list and incubation with the objective of eliminating other non-*Campylobacter* on an agar plate. For the enrichment step the various media that have been used for *Campylobacter* include m-Exeter broth, Peston broth, Bolton broth, and Park Sanders broth (Johnsen, et al. 2006, Oporto, et al. 2007, Hakkinen, et al. 2007, Bailey, et al. 2003, Thakur, et al. 2005, Wong, et al. 2007, Zanetti, et al. 1996, Taremi, et al. 2006, Bohaychuk, et al. 2006, Pezzotti, et al. 2003, Nye, et al. 2001, Petersen, et al. 2001, Whyte, et al. 2004, Ono, et al. 1999, Madden. 2002, Madden, et al. 1998, Zhao, et

al. 2001). The selection step involves an even wider selection of agar media including Campy-Cefex, mCampy-Cefex, CAMPY, Charcoal Cefoperazone Deoxycholate (CCDA), modified Charcoal Cefoperazone Deoxycholate (mCCDA), Campy-Line, Campy CVA, mCCDA with cefoperazone, amphotericin B, teicoplanin selective supplement (CAT agar), Preston plates, m-Exeter plates and Campylosel (Johnsen, et al. 2006, Oporto, et al. 2007, Hakkinen, et al. 2007, Bailey, et al. 2003, Thakur, et al. 2005, Wong, et al. 2007, Zanetti, et al. 1996, Taremi, et al. 2006, Bohaychuk, et al. 2006, Pezzotti, et al. 2003, Nye, et al. 2001, Petersen, et al. 2001, Whyte, et al. 2004, Ono, et al. 1999, Madden. 2002, Madden, et al. 1998, Zhao, et al. 2001, Bae, et al. 2005, Reid, et al. 2002, Ghafir, et al. 2007, Cloak, et al. 2001) (Table 1.1). Several comparative studies have evaluated the performance of different media in regard to sensitivity and selectivity ((Bolton, et al. 1983, Gun-Munro, et al. 1987, Endtz, et al. 1991, Chon, et al. 2011, Acke, et al. 2009, Kiess, et al. 2010, Ghazwan, et al. 2009). The media that did well in multiple studies were Preston agar, CCDA, mCCDA, CAT agar, Campy-line and Campy-cefex. CAT agar isolated *C. upsaliensis, C. lari,* and *C. helveticus* in addition to the *C. jejuni* and *C. coli* which helps identify possibly overlooked *Campylobacter* when using other methods. Campy-cefex was chosen for this project based on previous studies conducted under the direction of Wondwossen Gebreyes (Thakur, et al. 2005). The multitude of different media, amount of sample and amount of media all point to the obvious conclusion that there should be consistency in the way *Campylobacter spp.* of foodborne interest are cultured so that studies could be compared with more confidence.

The common identification tests include biochemical tests or gram stain and mobility. The bichemical tests for *Campylobacter* sp. include positive catalase and

oxidase. On a gram stain one would look for a gram negative corkscrew shaped rod and a wet mount would show darting mobility due to the singular polar flagellum. To distinguish between *C. jejuni*, *C. coli*, and *C. lari* the following biochemical tests are typically performed. *C. jejuni* will give a positive result for the hippurate hydrolysis, *C. lari* will be resistant to naladixic acid at 30 µg whereas the other two species will be susceptible. *C. coli* is different from the other two species in the lack of H_2S production. Latex agglutination and Enzyme Linked Immuno Sorbent Assays (ELISA) offer immunologic methods of differentiating species and genetically there are several primers that can be used to speciate *Campylobacter*. Biochemical tests are generally faster and cheaper than immunologic methods, but biochemical or immunologic methods are not used alone to speciate *Campylobacter* most studies will also include PCR to verify the species.

1.3 A novel method for culturing *Campylobacter*

A novel method of culturing *Campylobacter* named the preT-KB method was developed in 2004 (Baserisalehi, et al. 2004). The main difference between this method and the conventional method is that the new method does not include antibiotics or blood in the media and there is no enrichment therefore reducing the time and cost of culturing *Campylobacter* drastically. The theory is that *Campylobacter* are able to migrate out of the pellet quicker than the other competing bacteria and based on the mentioned study it appeared that *Campylobacter* was able to migrate out of the pellet quicker than *Proteus* and *E. coli*. The down side with this new method is if *Campylobacter* are stressed then they may not be able to migrate and if they were in very low numbers we may not be able to detect them without enrichment. The benefits to this method are that we could use

cheaper media and less labor to culture *Campylobacter* and since there are no antibiotics in the media we would have the potential to detect susceptible bacteria that would otherwise be missed.

1.4 Viable-but-nonculturable *Campylobacter*

Another challenge in the recovery of *Campylobacter* is that when stressed (by the pressence of oxygen, dehydration and cooler temperatures) it can enter a viable but nonculturable state (VBNC). *Campylobacter* can be induced to enter the VBNC state by suspending *C. jejuni* cells in sterile filtered surface water (Tholozan, et al. 1999, Cappelier, et al. 1999b). In order to resuscitate the injured cells most studies found the need to pass the cells through an animal model, a broth enrichment will not always be adequate to recover the bacteria (Cappelier, et al. 1999b, Cappelier, et al. 1999a, Oliver. 2005). The main difficulty in performing VBNC studies is to insure that there are no culturable cells inoculated into the animal system. It had been shown that dilution seems to be the best way to rule out the presence of culturable cells (Oliver, et al. 1995). When comparing a liquid medium to agar plates, the liquid medium has been reported to have better recovery rates for C*ampylobacter* that have been environmentally stressed (Bovill, et al. 1997, Mackey, et al. 1982). The addition of mucin to liquid medium can be used to further enhanced its ability to recover stressed *Campylobacter* cells (Bovill, et al. 1997, Mackey, et al. 1982). The most important conclusion is that when trying to recover stressed *Campylobacter*, conventional culture methods may be inadequate to identify a truly positive sample due to the possibility of the VBNC state.

1.5 *Campylobacter* prevalence in pig feces or intestine

There have been several studies that have reported the prevalence of *Campylobacter* in porcine feces. The prevalence of *Campylobacter* recovered from the feces of pigs from 5 farms in the Netherlands was 85% (Weijtens et al., 1993), and 79% in the Netherlands from swine intestinal contents at 3 harvest facilities (Oosterom, et al. 1985, Weijtens, et al. 1993). The prevalence of *Campylobacter* from rectal swabs collected post mortem in Norway was 100% from 114 pigs (Rosef, et al. 1983). Another study found the prevalence in the intestinal tract to be 100% in 121 pigs in Norway (Nesbakken, et al. 2003). A study conducted in 1985 in England from rectal contents taken post-mortem found a prevalence of 66% from 178 pigs (Manser, et al. 1985). A study in Italy found the prevalence to be 63.5% in rectal swabs prior to slaughter from 104 pigs (Pezzotti, et al. 2003) and a study in Spain observed a 52.9% herd prevalence out of 17 herds from pooled feces (Oporto, et al. 2007). A study conducted in North Carolina compared the prevalence of *Campylobacter* between conventionally raised swine (11 herds) and antibiotic free (5 herds) (Thakur, et al. 2005) and found no difference between the two groups at finishing; the conventional herds had a prevalence of 53% whereas the antibiotic free (ABF) herds had a prevalence of 55.8%. The results of these studies suggest that *Campylobacter* is very common in swine, and that intervention at the farm level appears impractical given that the lowest reported prevalence rate was 52.9%.

1.6 *Campylobacter* prevalence in cattle feces

There have also been several studies that reported the prevalence of *Campylobacter* in cattle feces. A study conducted in 1985 in England from rectal contents taken post-mortem found a prevalence of 23.5% from 309 cattle (animals that

were examined post-mortem by a veterinarian, not at slaughter whether they were finishing animals or dairy was not indicated) (Manser, et al. 1985) in Norway another study collected samples post-mortem and found a prevalence of 30% from a total of 804 cattle (there was no specification if the cattle were on pasture or in feedlots) representing 33 herds (Johnsen, et al. 2006) . A study conducted in Washington State found a prevalence of 31.6% for *C. jejuni* and 13.3% for *C. coli* in feedlot cattle from feces from 98 cattle on pasture at 2 ranches (Bae, et al. 2005). A study in the United Kingdom found a prevalence of 89.4% in beef cattle at slaughter from the lumen from 360 cattle from one abattoir (Stanley, et al. 1998). Other studies from around the world have found wide variation of carriage rates, from as low as 0.8% in Norway from fecal samples from 254 diary cows on farm (Rosef, et al. 1983), and 5% prevalence in California from beef cattle on pasture determined from fecal samples from 401 cattle representing 17 herds, (Hoar, et al. 2001). A higher prevalence of 19.5% was observed from fecal samples from 164 dairy cows in Portugal (Cabrita, et al. 1992). A 31.1% prevelance in Finland from 952 fecal samples from cattle in lairage from 12 slaughterhouses (Hakkinen, et al. 2007), 46.7% in Japan in 60 adult cattle from feces from 6 farms (Giacoboni, et al. 1993), 53.9% in Italy from 89 cattle rectal swabs prior slaughter (Pezzotti, et al. 2003), 58% in Australia from four farms and 100 cattle (Bailey, et al. 2003), 58.9% of herds from fecal samples in Spain out of 124 herds (Oporto, et al. 2007), and 83.7% in Canada from 380 fecal samples from cattle in an experimental setting (Inglis, et al. 2003). The cattle prevalence rates are more variable when compared to the swine prevalence rates. From these studies, beef cattle have higher prevalence when compared to dairy cattle. The beef cattle on pasture also had a lower prevalence when compared to beef cattle in feedlots.

Dairy cattle may shed *Campylobacter* less because they are older than beef cattle and their diet is different. Beef cattle on pasture may not be exposed to as high of a population density as are cattle in feedlots thus reducing the transmission of *Campylobacter*. The most common species of *Campylobacter* isolated from cattle is *C. jejuni,* which is also the most common pathogen in humans. The lower prevalence of *Campylobacter* in cattle indicates that it may be possible to adopt interventions for *C. jejuni* at the farm level.

1.7 *Campylobacter* prevalence during the slaughter process

Various studies have reported the prevalence of C*ampylobacter* along different stages of the slaughter process. A Danish study that included 600 pigs from 152 herds found the prevalence of *Campylobacter* to be 96% from feces, 66% on carcasses before chilling, 14% of carcasses one day after slaughter, and 0.5% from cuts of ham (Christensen, et al. 1994). Another study found the prevalence of *Campylobacter* to be 9% and 0% on the carcasses of pigs before and after chilling respectively in three different slaughterhouses, which may indicate that *Campylobacter* is sensitive to drying and does not survive well on exposed surfaces (Oosterom, et al. 1985). In Belgium a study found a prevalence of 17% (N=380) on the carcass (600cm^2) and 3.9% (N=355) on the resulting meat product (Ghafir, et al. 2007). This same study found a prevalence of 3.3% (N=60) on the carcass (400cm^2) and 5% (N=60) on minced beef. In a study conducted in Finland, 3.5% of cattle carcasses (brisket, the inner and outer thigh and the pelvic cavity) were contaminated with *Campylobacter* from 948 samples (Hakkinen, et al. 2007). One study investigated the presence of *Campylobacter* on the hide of grass-fed beef cattle at slaughter sampled immediately after bleed-out (Reid, et al. 2002). The

investigators did not recover any *Campylobacter* from the 90 samples. A study conducted in Northern Ireland investigated the prevalence of *Campylobacter* on beef carcasses, and *Campylobacter* was not detected in any of the 200 samples (Madden, et al. 2001). A study conducted in the United States surveyed 2089 steer/heifer carcasses and found a prevalence of 4.0% of *C. jejuni* (McNamara. 1995). A study in Australia surveyed 124 beef carcasses domestically and found one positive for *Campylobacter*, compared to exported carcasses where they found only one positive in 533 carcasses (Vanderlinde, et al. 1998). Another study compared isolation rates of *Campylobacter* on pig carcasses between samples collected pre-evisceration (103 conventional and 78 ABF) and samples collect post-evisceration (98 conventional and 88 ABF). They reported a significant difference with a higher isolation rate post-evisceration (Thakur, et al. 2005). This group also found a significant reduction of isolation of *Campylobacter* in the harvest process post chill. Blast chilling resulted in the most significant reduction compared to only 4°C chilling overnight.

1.8 *Campylobacter* prevalence in meat

The prevalence of *Campylobacter* in fresh meat products has been reported extensively. Several studies have been unable to isolate *Campylobacter* from pork or beef products (Bohaychuk, et al. 2006, Ono, et al. 1999, Madden, et al. 1998, Oosterom, et al. 1985). Other studies have shown the prevalence for *Campylobacter* in raw beef to range from 0.5% to 23.6% (Wong, et al. 2007, Taremi, et al. 2006, Pezzotti, et al. 2003, Whyte, et al. 2004, Whyte, et al. 2004, Ono, et al. 1999, Zhao, et al. 2001, Ghafir, et al. 2007, Cloak, et al. 2001, Svedhem, et al. 1981, Duffy, et al. 2001, Korsak, et al. 1998, Hong, et al. 2007, Osano, et al. 1999, Fricker, et al. 1989)). From pork, reported

prevalence has ranged from 1.3% to 18.4% (Zanetti, et al. 1996, Pezzotti, et al. 2003, Whyte, et al. 2004, Whyte, et al. 2004, Ono, et al. 1999, Zhao, et al. 2001, Ghafir, et al. 2007, Duffy, et al. 2001, Korsak, et al. 1998, Hong, et al. 2007, Fricker, et al. 1989). The meat products had a low prevalence regardless of sample size and sample amount tested. These studies indicate that more investigation is needed to identify effective interventions to prevent contamination of fresh meat products with *Campylobacter*.

1.9 *Campylobacter* prevalence from different sample types

There are also other less common samples from which *Campylobacter* has been reported to be isolated. A study conducted in Ontario, Canada found higher isolation rates of *Campylobacter jejuni* from the gall bladders (33%), large intestines (35%), and small intestines (31%) than from the livers (12%), or the lymph nodes (1.4%) in cattle (Garcia, et al. 1985). Another study looked at the prevalence of *Campylobacter* along the various stages of the digestive tract in cattle and found the following isolation rates, rumen (30%), true stomach (0%), small intestine (60%), large intestine (0%), and caeca (0%) (Stanley, et al. 1998). This same study reported that calves had nearly 100 times higher number of *Campylobacter* in their intestinal samples than beef cattle sampled at slaughter. Others have reported higher isolation rates in calves and piglets when compared to more mature animals, which is important for food safety.

1.10 *Campylobacter* quantification

There are few studies that have investigated the quantity of *Campylobacter* in carcass and meat samples, and only one investigating fecal material. There also are not any available reports quantifying *Campylobacter* in fecal material, carcass and meat all observed in the same study. In the US, a study found a mean *C. jejuni* concentration of

0.1 MPN/cm^2 (McNamara. 1995) on steer carcasses, and another reported that the counts were less than 100 CFU per carcass with one exception of a carcass having 460 CFU per carcass in pigs (Oosterom, et al. 1985). In studies analyzing fresh meat samples, one study found only one of four minced beef samples to be contaminated with *C. jejuni* above the detection level of 5 to 10 CFU per g (Cloak, et al. 2001). Another study found 8 out of 230 samples of beef to be contaminated at the level of less than 0.3 CFU per g (Wong, et al. 2007). This same study found pork contaminated at the rate of 18 per 230 with 17 that were less than 0.3 CFU per gram and one that was between 0.3 and 1.0 CFU per g. Another study found concentrations of *Campylobacter* in pork carcasses were 1 CFU per 600cm^2 (83%), 1-249 CFU per 600cm^2 (15%) and greater than 250 CFU per 600cm^2 (2%) (Ghafir, et al. 2007) This same study found the concentrations in meat to be, 93% were less than 1CFU per 25g, 7% 1 to 249 CFU per 25g and 0.5% greater than 250 CFU per 25g. Another study of beef cattle going to slaughter found an average CFU per g^- of 6.1 x 10^2 in pre-fecal material (Stanley, et al. 1998). Even though the concentration in the intestine of cattle was very high these studies indicate that the meat samples did not have high concentrations and most did not contain an infectious dose if a person ate a reasonable amount of meat. None of these studies however followed the same animals through the slaughter process to see if there was an association between concentrations during the process and those on the meat.

1.11 Blast chilling

Various methods have been investigated to attempt to eliminate or reduce the amount of viable *Campylobacter* on carcass and meat products. Previous research has shown a great reduction of *Campylobacter* contamination of pig carcass's following blast

chilling (Oosterom, et al. 1985, Bracewell, et al. 1985, Nesbakken, et al. 2008). Two other studies found that blast chilling was significantly more effective at reducing *Campylobacter coli* from pig carcass's than conventional chilling (Chang, et al. 2003). Other studies have shown that *Campylobacter* cells are greatly reduced after freeze thaw cycles (Stern, et al. 1985, Stead, et al. 2000, Humphrey, et al. 1985, Hanninen. 1981, Moorhead, et al. 2002, Georgsson, et al. 2006) Several studies have shown there to be no decrease in survival of *Campylobacter* left at 4°C for up to 8 days inoculated on raw meat (Svedhem, et al. 1981, Pintar, et al. 2007, Blankenship, et al. 1982, Solow, et al. 2003, Dykes, et al. 2001, Davis, et al. 2007, Bostan, et al. 2001) . Another study did see a decline from 0.31 to 0.63 log CFU/g on chicken skin from 3 to 7 days (Bhaduri, et al. 2004). The use of gamma irradiation (GI) to sterilize (radappertization) or pasteurize (radurization) foods was studied as early as the 1960's (Coleby, et al. 1961). The safety of the food after the radiation is not a concern, however consumers frequently avoid products exposed to radiation making them difficult to market. All other means of decontaminating fresh meat products have not proven effective including steam or hot water dips or sprays, electromagnetic waves, high intensity pulsed electric field, oscillating magnetic field, pulsed light, air ions, high pressure processing, ultrasonic energy, and natural antimicrobials (Dinçer, et al. 2004).

1.12 *Campylobacter* PCR from feces

Some investigators argue that the best way to determine prevalence of *Campylobacter* is to perform PCR on DNA extractions of samples without culturing. One study investigated an internal control that was added to the feces before DNA extraction to determine if PCR inhibitors had been removed ((Inglis, et al. 2004)). The

initial study (Inglis, et al. 2003) using the Qiagen stool kit found that in only two out of 382 samples were they unable to detect the positive internal control. The rationale they gave for the importance of real time PCR for detecting *Campylobacter* in feces is (i) the rapidity of the method; (ii) the lack of discrimination against rarely occurring species; (iii) the immediate delineation of species (rather than a reliance on colony selection, sub-culturing, and subsequent identification); (iv) PCR is not influenced by the physiological requirements of the bacteria (e.g., temperature and atmosphere); (v) the ability o process small samples (i.e., ~200mg); and (vi) the logistical advantaged of not requiring specialized culturing equipment, viable bacteria (e.g., loss of viability during transportation), or immediate processing of samples. Epidemiologically, the major disadvantage to only using PCR is that there is not an isolate available to further characterize for clonality or antimicrobial resistance patterns.

1.13 *Campylobacter* speciation

There have been differences in the species of *Campylobacter* isolated from cattle and pigs. A study conducted in Belgium found the prevalence of *C. jejuni* among samples positive for *Campylobacter* to be 75% (n=9) in pork and 100% (n=5) in beef. A different study found two species in pork, *C. coli* n=2 and *C. lari* n=1 (Ghafir, et al. 2007). Another study found only *C. jejuni* in beef steaks and a mixture of *C. jejuni* (2 out of 11) and *C. coli* (9 out of 11) in pork chops (Zhao, et al. 2001). A study conducted in England found 100% *C. coli* in pigs and 75% *C. jejuni* in cattle. Five isolates from cattle were *C. fetus* subsp. f*etus* (Manser, et al. 1985). A study in Canada using real time PCR found out of the 380 fecal samples tested, 318 were positive for *Campylobacter* DNA and the prevalence of the different species were as follows, 49.2% *C. lanienae*, 37.9% ,*C.*

jejuni, 7.9% *C. hyointestinalis* and 0.5% *C. coli* (Inglis, et al. 2003). A study in Italy observed *Campylobacter* from beef cattle to be 22.2% *C. jejuni*, 25.9% *C. coli* and 51.9% other thermophilic *Campylobacter* species (Pezzotti, et al. 2003). In the same study the results for pigs were 1.3% *C. jejuni*, 63.5% *C. coli* and 35.2% other *Campylobacter*, most of which were identified as *C. hyointestinalis*. In meat samples, 2 isolates from beef were *C. jejuni* and of the 18 isolates from pork 7 were *C. jejuni* and 10 were *C. coli*, while a final isolate was another unidentified *Campylobacter* species. A study in Norway collected intestinal samples post-mortem and reported the prevalence of *Campylobacter* species. They reported that *C. jejuni* was isolated from 26% of the cattle and *C. coli* was isolated from 3%, and an unidentified *Campylobacter* was isolated from 2% of the cattle (Johnsen, et al. 2006). A study in Finland yielded a total of 296 *Campylobacter* isolates from 952 post mortem rectal samples, and 33 *Campylobacter* isolates from 948 carcass swab samples. From the fecal samples *C. jejuni* was detected in 60%, presumptive *C. hyointestinals* in 33.2% and *C. coli* in 6.8%. For the carcass samples *C. jejuni* was detected in 87.9%, presumptive *C. hyointestinalis* 6.1% and *C. coli* 6.1% (Hakkinen, et al. 2007). The different recovery rates for different species might indicate that certain species of *Campylobacter* are host adaptive. Also, it appears that PCR will reveal a higher diversity of strains compared to culture which may be due to *Campylobacter* competition between strains and hardiness in the different media.

1.14 Antimicrobial Resistance

Antibiotic resistance is becoming an increasing phenomenon among *Campylobacter* and other food-borne disease organisms. Fluoroquinolones (e.g., ciprofloxacin) are commonly used in adult humans to treat Campylobacteriosis.

Fluoroquinolones (e.g., enrofloxacin) are also used in food animals to treat various bacterial infections. Prior to 1995 fluroquinolones were not approved for use in food animals. In 1995, sarafloxacin was approved by the FDA and in 1996 enrofloxacin was approved both in poultry for the treatment of respiratory disease, and each were given through the water to the whole flock. Prior to 1995 very little fluoroquinolone resistance was found in *Campylobacter* sp. isolated from humans. By 1999 18% of *Campylobacter* spp. isolated from human clinical cases were resistant to ciprofloxacin, and by 2008 the resistance was up to 23% (Anonymous 2010). In 2000 the FDA requested a hearing for the removal of fluroquinolones from the use in poultry. Before the FDA request was made, Abbott Laboratories voluntarily removed sarafloxacin from the market for poultry so the hearing was only applicable to enrofloxacin (Bayer Corporation). Bayer did request the hearing so in 2002 the hearing officially started and the two judges agreed to prohibit the use of enrofloxacin in poultry effective September 2005. This was the first time a drug used in animals was banned due to a link of antimicrobial resistance in humans ((Nelson, et al. 2007)).

Antibiotic resistance of isolates is not a major focus of this dissertation, but it is worth mentioning some of the studies that have investigated antibiotic resistance, especially in *C. coli.* A study in Denmark found that more macrolide-resistance was present in *C. coli* isolated from swine (79%) than from *C. coli* isolates from broilers (18%) and humans (14%) (Aarestrup, et al. 1997). This same study observed a higher resistance to streptomycin in *C. coli* isolated from swine (48%) than from broilers (6%) and humans (0%). In a study of retail meat products, the investigators observed that 94% of the *Campylobacter* isolates were resistant to at least one antibiotic. Resistance to

tetracycline (82%), doxycycline (77%), erythromycin (54%), nalidixic acid (41%) and ciprofloxacin (35%) were the most common antibiotics in poultry isolates. None of the isolates were resistant to gentamicin (Ge, et al. 2003)

A study conducted in swine found that *C. coli* isolates had the highest frequency of resistance to tetracycline (66.2%) and erythromycin (53.6%) which may reflect the common use of these antimicrobials in swine production (Thakur, et al. 2005). This study also compared *Campylobacter* isolates between conventional systems and antibiotic free systems and found a significantly higher percentage of tetracycline and erythromycin resistant strains within the conventional system. They also observed that 78.9% of the isolates were pansusceptable.

A study in cattle of the antimicrobial resistance patterns in *C. jejuni* and *C. coli* found that for ciprofloxacin, nalidixic acid and trimethoprim/sulfamethoxazole *C. coli* exhibited significantly higher prevalence of resistance (Englen, et al. 2005). Overall, food animal isolates are resistant to many classes of antibiotics and *C. coli* typically shows higher resistance to individual antibiotics when compared to *C. jejuni*, also *C. coli* in swine appear to exhibit higher resistance to antimicrobials when compared to cattle and poultry isolates.

1.15 Genotyping

Various methods have been investigated to genotype *Campylobacter*. A study evaluating typing methods found that when comparing pulsed field gel electrophoresis (PFGE) to random amplification of polymorphic DNA (RAPD) there was some level of agreement when comparing strain differentiation and grouping but in 40% of the isolates the two methods disagreed (Nielsen, et al. 2000). This same study found the Penner

serotyping method to be useful for typing large numbers of isolates to obtain a coarse grouping. Another study (Dingle, et al. 2001) found that multi locus sequence typing (MLST) was an appropriate method of typing for *Campylobacter*. The study demonstrated that MLST (i) discriminates among *C. jejuni* isolates effectively and (ii) generates data that can be applied to the investigation of the population structure and evolutionary mechanisms in this organism. The advantages of MLST include high discrimination, good reproducibility, simplicity of interpretation and the data can be compared among laboratories via the internet.

Another study utilized amplified fragment length polymorphism (AFLP) to determine clonality of *Campylobacter jejuni* from cattle and humans (Johnsen, et al. 2006). The results indicated that in one herd three distinct genetic clones were consistently isolated over several months. There was also genetic similarity between bovine and human strains. The highest carriage rates were isolated from calves rather from adult cattle. Three molecular typing methods were used to characterize a set of 180 *C. jejuni* and 4 *C. coli* strains in another study (Schouls, et al. 2003). The techniques used were AFLP, MLST and sequence analysis of a genomic region with short tandem repeats termed, clustered regularly interspaced short palindromic repeats (CRISPRs). All of the methods were suitable for *Campylobacter* with high discriminatory power and similar groupings. The CRISPRs method though had a disadvantage in that 26% of the *Campylobacter* as non-typeable because they only contained a single repeat sequence or the lack of an amplifiable CRISPRs locus. Between the other two methods the authors recommended MLST due to the ability to share results between laboratories and because it provides solid sequencing data. By using a statistical method known as group

separation the authors observed that based on the AFLP patterns, strains isolated from humans and poultry were most closely related to other strains from humans and poultry. When looking at cattle they found that cattle were most closely related to strains from humans and only a third were related to other cattle strains. There was not an association between cattle and poultry isolates. They hypothesized that humans are exposed to *Campylobacter* from similar but more diverse sources than cattle. The authors also conclude that typing of *Campylobacter* strains is useful for identification of outbreaks but may not be as useful for source tracing and global epidemiology due to the carriage of strains of multiple types and an extremely high diversity of strains from animal sources.

An interesting study used a genotyping technique to compare 111 *C. jejuni* strains from various agricultural, human disease outcomes and environmental sources (Champion, et al. 2005). The authors utilized a microarrary based on the sequence of NCTC11168 (*C. jejuni*). By use of Bayesian phylogeny the isolates were grouped into clades which resulted in the identification of two distinct clades. One clade comprised 31 of the 35 livestock isolates (bovine, chicken, and ovine) and the other comprised further clades of environmental isolates. The majority of the human isolates (55.7%) grouped in the non-livestock clade which suggests that some *C. jejuni* infectious may be from non-livestock sources. Another study (Hakkinen, et al. 2007) found a high degree of diversity among bovine *C. jejuni* isolates employing PFGE. When comparing the PFGE patterns from fecal samples and carcass samples they found that some subtypes commonly detected in fecal samples were not isolated from carcasses. Also, one of the most common subtypes in the carcass samples was not isolated from the feces. The authors

hypothesized that subtypes exist which are poor competitors in the intestinal tract but may have an enhanced ability to survive the stresses of the surface of the carcass.

Table 1.1 Composition of the media used in the experiments

Medium	Base per Liter	Supplement per Liter	Species Recommended
Campy-Cefex	Brucella Agar [Pancreatic digest of casein (10 g), peptic digest of animal tissue (10 g), dextrose (1 g), yeast extract (2 g), sodium chloride (5 g), sodium bisulfite (0.1 g), agar (15 g)], ferrous sulfate (0.5 g), sodium bisulfite (0.2 g), sodium pyruvate (0.5 g)	Laked horse blood (50 ml), cefoperazone (33 mg), cycloheximide (0.2 g)	*C. jejuni, C. coli*, and *C. lari*
mCampy-Cefex	*Brucella* agar (43 g), ferrous sulfate (0.5 g), sodium bisulfite (0.2 g), sodium pyruvate (0.5 g)	Lysed horse blood (50 ml), cefoperazone (33 mg), amphotericin B (2 mg)	*C. jejuni, C. coli*, and *C. lari*
CAMPY	*Brucella* agar (43 g) pyruvate (0.25 g), sodium metabisulfite (0.25 g), ferrous sulfate (0.25 g)	Lysed horse blood (70 ml), polymyxin B sulfate (1 mg), trimethoprim (0.01 g), vancomycin (0.01 g), amphotericin B (2 mg), novobiocin (0.05 g), sodium	*C. jejuni*
CCDA	Nutrient broth no. 2 [beef extract (10 g), peptone (10 g), sodium chloride (5 g)], bacteriological charcoal (4 g), casein hydrolysate (3 g), sodium desoxycholate (1 g), ferrous sulfate (0.25 g), sodium pyruvate (0.25 g), agar (12 g)	Cefoperazone (32 mg), cycloheximide (100 mg)	*C. jejuni, C. coli*, and *C. laridis*

Continued

Table 1.1 Continued

Medium	Base per Liter	Supplement per Liter	Species Recommended
mCCDA	Nutrient broth no. 2 [beef extract (10 g), peptone (10 g), sodium chloride (5 g)], bacteriological charcoal (4 g), casein hydrolysate (3 g), sodium desoxycholate (1 g), ferrous sulfate (0.25 g), sodium pyruvate (0.25 g), agar (12 g)	Cefoperazone (32 mg), amphotericin B (10 mg)	*C. jejuni*, *C. coli*, and *C. laridis*
Campy-Line	*Brucella* agar (43 g), ferrous sulfate (0.5 g), sodium bisulfite (0.2 g), sodium pyruvate (0.5 g), alpha-ketoglutaric acid (1 g), sodium carbonate (0.6 g)	Hemin (10 mg), polymyxin B sulfate (0.35 mg), trimethoprim (5 mg), vancomycin (10 mg), cycloheximide (100 mg), cefoperazone (33 mg), triphenyltetrazolium chloride (200 mg)	*C. jejuni*, *C. coli*, and *C. lari*
Campy Thioglycolate medium	Pancreatic digest of casein (17 g), Papaic Digest of Soybean Meal (3 g), dextrose (6 g), sodium chloride (2.5 g), sodium thioglycollate (0.5 g), agar 1.6 g, L-Cystine (0.25 g), sodium sulfite (0.2 g)	amphotericin B (2 mg), cephalothin (15 mg), trimethoprim (5 mg), vancomycin (10 mg), polymyxin B (2,500U)	*C. jejuni*
Campy-CVA	Brucella agar (43 g)	cefoperazone (20 mg), vancomycin (10 mg), amphotericin B (2 mg), defribinated sheep blood (50ml)	*C. jejuni*

Continued

Table 1.1 Continued

Medium	Base per Liter	Supplement per Liter	Species Recommended
CAT agar	Nutrient broth no. 2 [beef extract (10 g), peptone (10 g), sodium chloride (5 g)], bacteriological charcoal (4 g), casein hydrolysate (3 g), sodium desoxycholate (1 g), ferrous sulfate (0.25 g), sodium pyruvate (0.25 g), agar (12 g)	cefoperazone (8 mg), Teicoplanin (4 ml), Amphotericin B (10 mg)	*C. jejuni, C. coli, C. upsaliensis, C. lari, and C. helveticus*
Preston Broth	Nutrient broth no. 2 (25 g), sodium pyruvate (0.25 g), sodium metabisulphite (0.25 g) ferrous sulphate (0.25 g)	polymyxin B (5,000U), rifampicin (10mg), trimethoprim (5 mg), cycloheximide (100 mg) lysed horse blood (50ml)	*C. jejuni*, and *C. coli*
Preston plates	"Lab Lemco" powder (10 g), peptone (10 g), sodium chloride (5 g), agar (12 g), sodium pyruvate (0.25 g), sodium metabisulphite (0.25 g), ferrous sulphate. (0.25 g)	polymyxin B (5,000U), rifampicin (10mg), trimethoprim (5 mg), cycloheximide (100 mg) lysed horse blood (50 ml)	*C. jejuni*, and *C. coli*
Bolton Broth	Meat peptone (10 g), Lactalbumin hydrolysate (5 g), yeast extract (5 g), sodium chloride (5 g), Alpha-ketoglutaric acid (1 g), sodium pyruvate (0.5 g), sodium metabisulphite (0.5 g), sodium carbonate (0.6 g), haemin (0.01 g)	cefoperazone (20 mg), vancomycin (20 mg), trimethoprim (20 mg), cycloheximide (50 mg), laked horse blood (50 ml)	*Campylobacter sp.*

Continued

Table 1.1 Continued

Medium	Base per Liter	Supplement per Liter	Species Recommended
Park Sanders broth	brucella broth (28 g), sodium citrate (1 g), sodium pyruvate (0.25 g)	vancomycin (20 mg) trimethoprim lactate (20 mg) horse blood (11.8 ml)	*Campylobacter sp.*
m-Exeter broth	25 g Nutrient Broth No.2 sodium metabisulphate (0.5 g) sodium pyruvate (0.5 g) iron sulphate (1 g)	cefaperazone (15 mg) polymyxin B (5,000U), rifampicin (10mg), trimethoprim (5 mg), amphotericin B (10 mg) lysed horse blood (50 ml)	*Campylobacter sp.*
m-Exeter plates	25 g Nutrient Broth No.2 sodium metabisulphate (0.5 g) sodium pyruvate (0.5 g) iron sulphate (1 g) agar (12 g)	cefaperazone (15 mg) polymyxin B (5,000U), rifampicin (10mg), trimethoprim (5 mg), amphotericin B (10 mg) lysed horse blood (50 ml)	*Campylobacter sp.*
KB Medium	tryptone (10 g), yeast extract (5 g), lactose (10 g), NaCl 5 g, Na_2HPO_4 (0.8 g), phenol red (0.035 g), malachite green (1% solution 5 ml), glycerol (5 ml), agar (15 g)	vit-E solution (140 mg/ml in 10% Tween 80) (5 ml)	*Campylobacter sp.*
Campylosel	Proprietary	cefaperazone (32 mg), vancomycin (10 mg), amphotericin B (3 mg)	*C. jejuni* and *C. coli*

Modified from (Hakkinen, et al. 2007)

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Chapter 2 Comparison of Direct Dilution, Most Probable Number estimation, and Quantitative real-time PCR for enumeration of *Salmonella* and *Campylobacter* in spiked cattle and pig samples

2.1 Introduction

Salmonella and *Campylobacter* are major foodborne pathogens in the United States (Scallan, et al. 2011). Swine and cattle frequently shed these potential pathogens asymptomatically, which presents a food safety risk when the carcass becomes contaminated with feces during processing. Quantitative measures of foodborne safety risk are rarely reported in the literature, most likely as a consequence of the substantial labor and media requirements of traditional culture based methods for determining pathogen concentration. Quantitative measures of contamination may provide more useful measures to evaluate interventions and to collect data for public health risk assessments. The objective of this study was to compare estimates of *Salmonella* and *Campylobacter* concentrations using most probable number (MPN) method, direct dilution, and quantitative real-time PCR (QPCR) in spiked fecal, hide, carcass, and meat samples.

2.2 Materials and Methods

2.2.1 Preparation of inoculum

The strain of *Salmonella* used for spiking was *Salmonella enterica* subsp. *enterica* ATCC 13076. The *Campylobacter* strains used for spiking were *Campylobacter jejuni* subsp. *jejuni* ATCC 33560 and *Campylobacter coli* ATCC 49941. *Salmonella enterica* colonies were grown in nutrient broth and incubated overnight on a shaker at 37º C. *Campylobacter sps.* Colonies were grown in Brucella broth (Becton, Dickinson and Company (BD), Sparks, MD) in microaerophilic (O_2 6 – 16%, and CO_2 2 – 10% Campy EZ Gas Packs, BD) conditions.

2.2.2 Inoculation and sampling of tissues

Using the 0.5 McFarland Standard and Nephlometer (Trek Diagnostic Systems, Westlake OH), the bacteria were diluted to a concentration of 10^8 CFU/mL, and subsequent dilutions were performed to achieve a dilution series from 10^8 CFU/mL to 10^1 CFU/mL. Based on the weight of the feces, meat, or hide and carcass area the amount of bacteria was calculated to inoculate the sample at 1mL of the dilution per gram for the meat and feces, and 1mL per cm^2 for the hide and carcass simulation. The fecal samples for inoculation were collected from live animals and processed within 8 hours of collection. The hides of cattle were swabbed using a pre moistened sponge (hydrated-sponge, 3M, Saint Paul, MN) while still alive and processed within 8 hours of collection. A beef roast purchased from a local grocery store was cut into slices and the surface was inoculated to simulate carcass contamination. Ground meat for inoculation was also purchased from a local grocery store. For all of the samples the bacteria was added to the sample in the laboratory based on either the weight or surface area of the sample. For each of the spiking experiments, the diluted bacteria was inoculated onto agar plates

before and after adding the control strains to verify the sterility of the media and to verify the quantity of bacteria.

2.2.3 Procedures for direct dilution

Campylobacter coli and *Campylobacter jejuni* was spiked on the samples from 1 x 10^1 to 1 x 10^8 CFU/mL for the bovine feces, and porcine feces. The fecal samples for inoculation were processed by adding 1g of feces to 9ml of BPW. Three dilutions were created by pipetting 1ml of the initial 10^{-1} dilution and adding to 9ml of BPW, repeatedly to a maximum dilution of 10^{-4}. One-hundred microliters from each dilution was spread-plated in duplicate on Campy-Cefex (Oyarzabal, et al. 2005) agar and incubated under microaerophilic (O_2 5-15% and CO_2 5-12%, Campy GasPak, BD) conditions for 48h at 42° C. *Campylobacter* suspect colonies were then counted.

2.2.4 Procedures for MPN estimation

2.2.4.1 *Salmonella*

Salmonella was inoculated onto the samples using concentrations ranging from 1 x 10^1 to 1 x 10^8 CFU/mL for the bovine feces, porcine feces, carcass, and ground beef. The fecal samples were processed for inoculation by weighing 4g (cattle) or 10g (swine) of feces and adding to 36ml (cattle) or 90ml (swine) of Tetrathionate broth (TTB, Becton Dickenson) in a three tube-five serial 10-fold dilution MPN method. The dilutions were prepared by pipetting 4ml or 10ml from the 10^{-1} dilution, which was then mixed in the next tube with 36ml or 90ml of TTB to make the 10^{-2} dilution and this was continued to a final dilution of 10^{-5}. The fecal samples incubated at 37° C for 24h. A 100µl aliquot from each tube was plated onto XLT4 (Becton Dickenson) agar plates and incubated for another 18 – 24h at 37° C. The sponge from the simulated carcass swab

(roast) was cut into fourths and mixed with 90ml of BPW for the 10^{-1} dilution. Ten milliliters was then added to each of the other three tubes containing 90ml of BPW repeatedly to create the seven dilutions. The tubes were incubated for 24h 37° C. One-hundred microliters from each tube was then added to 10ml of Rappaport-Vassiliadis R10 (RV, Becton Dickenson) broth and incubated at 42° C for 18 – 24h. An aliquot of 100µl from each tube was plated onto XLT4 agar plates and incubated for another 18 – 24h at 37° C. Ten grams of ground beef was mixed with 90ml of BPW and 10ml of this mixture was added to 90ml of BPW to make the 3 tube 4 dilution MPN. The samples then followed the protocol for the carcass swabs. The MPN was calculated using the Excel sheet developed by FDA guidelines (Garthright, et al. 2003).

2.2.4.2 *Campylobacter*

Campylobacter jejuni and *Campylobacter coli* was spiked with the samples from 1 x 10^1 to 1 x 10^6 CFU/mL for the carcass and ground beef, and from 1 x 10^1 to 1 x 10^5 CFU/mL for the hide samples. The hide samples were processed by mixing the sponge with 30ml of Bolton broth (EMD). Ten milliliters of the Bolton broth mixture with the sponge was inoculated into three tubes, and 1 ml of this mixture was taken and added to 9ml of Bolton broth to make the next dilution to create the 3 tube x 4 dilution series. The tubes were incubated under microaerophilic conditions for 48h at 42° C. One-hundred microliters from each dilution was spread-plated onto Campy-Cefex plates and incubated under microaerophilic conditions for 48h at 42° C. The 5 sponges from the carcass swab were pooled and mixed with 150ml of Bolton broth. A 30ml aliquot of the pooled carcass swab sample was added to each of three tubes and 3ml from

the 10^{-1} dilution was added to 27ml of Bolton broth to make a 3 tube x 4 dilution MPN. The samples then followed the same protocol as described for the hide samples. Ten grams of ground meat was mixed with 90ml of Bolton broth and 10ml of this mixture was added to 90ml of Bolton broth to make the 3 tube, 4 dilution series. The samples then followed the protocol for the hide samples and carcass swabs from this point on.

2.2.5 DNA extraction procedures

For Q-PCR, a fecal scoop (Sarstedt stool tube) of ~200mg was used to obtain a fecal sample pre- and-post spiking. For the meat samples, a 1g sample was collected and placed in 40mL of BPW. The carcass swab used for PCR was placed in 80mL of BPW. DNA was extracted from each sample using different methods. DNA from the fecal scoops was extracted using the Qiagen (Valencia, CA) QIAamp DNA Stool Mini Kit according to manufacturer's instructions. A two step centrifuge method (for ground meat) of initially 300 rpm to pellet large meat pieces, then the supernatant was poured off to be centrifuged for 3,000 rpm to pellet the bacterial cells. The DNA was then extracted from the pellet using the DNeasy Tissue Kit from Qiagen. Forty milliliters from the carcass rinse was centrifuged at 3,000rpm to pellet the bacterial cells then the DNA was extracted from the pellet using the DNeasy Tissue Kit as well.

2.2.6 Procedures for QPCR

2.2.6.1 *Salmonella*

Salmonella was spiked with the samples from 1×10^1 to 1×10^8 CFU/mL for the bovine feces, porcine feces, carcass and ground beef. Real time quantitative PCR was performed on the Mx3000P. All samples were tested in triplicate. The reactions were at a 25µl final volume. The reaction contained 12.5µl of Brilliant SYBR Green

QPCR master mix (Stratagene, CA) or Qiagen QuaniTect SYBR Green PCR kit, 1.25µl of extracted DNA (~100ng) and 0.5µM of each primer (Table 2.1). Cycling parameters were different based on which primer set was evaluated (Table 2.2).

2.2.6.2 Campylobacter

In this study the quantitative PCR for *Campylobacter* was not evaluated because we were unable to successfully get both probes (one for *C. jejuni* and one for *C. coli*) to work simultaneously together. Therefore, we were unable to compare QPCR to MPN for *Campylobacter*.

2.2.7 Statistical Analysis

The comparisons of the different quantitative methods (direct dilution, MPN and QPCR) between the pre and post spiked concentrations were analyzed by the Spearman's rank correlation coefficient using STATA (Intercoolated STATA 9, StataCorp, College Station, TX) to determine the assocaition between inital concetration and recovered concetration. When the MPN method yielded a too numerous to count result a number that was larger than the highest number generated by MPN for each different sample set was assigned and used consistently within the sample set.

2.3 Results

2.3.1 *Salmonella* MPN

All of the samples reached the limit of the MPN so there were TNTC (too numerous to calculate, since all the plates were positive) values. The results indicate that there was a strong positive correlation between pre and post spiking concentrations for

the MPN results from the bovine and porcine fecal samples (Table 2.2, Figure 2.6, 2.7 and 2.8).

2.3.2 *Campylobacter* quantification direct dilution

Spearman rank coefficient values revealed only the bovine feces for *C. coli* was significant for a strong positive correlation between initial spiking concentrations and post-spiking values (Table 2.4, Figure 2.1 and 2.2).

2.3.3 *Campylobacter* MPN

All of the sample types reached the limit of the MPN (except ground beef for *Campylobacter jejuni* and the hide sample for *Campylobacter coli*) so there were TNTC (too numerous to calculate, since all the plates were positive) values. These results indicate that there was a strong positive correlation between the pre and post spiked samples for hide, carcass and ground beef samples, except the hide sample spiked with *C. coli* (Table 2.5, Figure 2.3 and 2.4).

2.3.4 QPCR *Salmonella*

The evaluation of the primer sets found that the Styinva-JHO-2 (Hoorfar, et al. 2000) preformed the best with our conditions and machine based on the dissociation curve and efficiency of the reaction. The results indicate that there was a strong positive correlation between the pre and post spiking samples for all the QPCR results (Table 2.3 and Figure 2.5).

2.4 Discussion

The results for QPCR indicated there was a strong positive correlation (Spearman's $R \geq 0.95$, $p \leq 0.0003$) between the pre- and post-spiking samples for all the

sample types tested. *Salmonella* QPCR results for bovine feces and meat were both lower than expected based on the initial spiking concentration. One explanation is that there could be inhibitors present in the sample, which prevented the *Salmonella* from being cultured or detected on PCR. Another explanation is that there could have been an error made during the processing, DNA extraction or PCR. The quantitative PCR results for *Salmonella* indicated that carcass swab samples observed values very similar to the initial spiked concentration; however, porcine feces results were one log lower than expected, meat was 2 to 3 logs lower and cattle feces was about 4 Log 10 lower. These observations could indicate that there were PCR inhibitors present in some sample matrices and not in others and present in varying degrees. Although another research team demonstrated that the Qiagen stool kit effectively removed PCR inhibitors in bovine feces (Inglis, et al. 2003). There could also have been human error in the PCR set-up or spiking aspects.

Salmonella MPN results indicated there was a strong positive correlation (Spearman's $R \geq 0.70$, $p \leq 0.054$) between pre and post spiking concentrations for the MPN results from the bovine, and porcine, samples and a non significant result for the carcass and meat samples. This result could indicate that *Salmonella* may reproduce and survive better in the presence of fecal material, or that the culture method worked better with the fecal samples. Also, the simulated carcass sample was swabbed so the bacteria may adhere to the meat.

Campylobacter direct dilution results indicated a good correlation between the pre- and post-spiking samples for the higher values but the lower limit may have been influenced by the possibility that the initial feces may not have been *Campylobacter* free.

The feces that was collected was initially cultured to determine if it was *Campylobacter* free but culture is not very sensitive therefore this may not have been the best measure. This seems like a possible explanation when the MPN in feces for *Salmonella* did not show this same trend although there is a difference in the methodology between direct dilution and MPN so they cannot be directly compared.

Campylobacter MPN results indicated there was a strong positive correlation (Spearman's $R \geq 0.87$, $p \leq 0.054$) between the pre- and post-spiked samples for hide, carcass and ground beef samples and all were significant except the hide sample spiked with *C. coli*. *Campylobacter* MPN results were much lower than expected based on the spiking concentration. Various explanations can be raised for this observation. The MPN may not be the best method for *Campylobacter* or the enrichment media may not be the best. There also could have been human error when adding the spiking concentrations. The MPN should have also been adjusted so that there were not as many too numerous calculate results this could have been addressed by increasing the number of dilutions. This would have increased the amount of cost and labor though to an already expensive and high labor project. One limit to *Campylobacter* MPN is that we did not distinguish between *C. coli* and *C. jejuni* colonies on campy-cefex plates. Therefore, if the starting feces did contain some *C. jejuni* or *C. coli* colonies then those may have been counted as well as the strain that was spiking. To avoid this one could perform PCR or biochemical tests to distinguish between the two species on multiple colonies from each plate used to calculate the MPN. This would also incur more cost and labor.

The overall findings of this study was the QPCR indicated there was a strong positive correlation between the pre- and post-spiking samples for all the sample types tested, *Salmonella* MPN results indicated there was a strong positive correlation between pre and post spiking concentrations for the MPN results from the bovine, and porcine, fecal samples, *Campylobacter* direct dilution revealed only the bovine feces for *C. coli* was significant for a strong positive correlation between initial spiking concentrations and post spiking values, and *Campylobacter* MPN results indicated there was a strong positive correlation between the pre- and post-spiked samples for carcass and ground beef samples. QPCR provided good correlation between pre- and post-spiked samples for *Salmonella* and another quantitative method appears to be needed for *Campylobacter*. This study should help to clarify some of the existing methods for quantification and open our eyes to develop a better method for *Campylobacter.*

2.5 References

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Table 2.1 Oligonucleotide primers evaluated in the Q-PCR assay for *Salmonella* spiked samples

Primer	Sequence (5'-3')	Bacteria Species	Target gene	Reference
InvA-1F InvA-1R	TTCCATTACCTACCTATCTGG TTGATT GAACGACCCCATAAACACCA A	*Salmonella sp.*	*inv* A	(Iijima, et al. 2004)
Styinva-JHO-2-left Styinva-JHO-2-right	TCGTCATTCCATTACCTACC AAACGTTGAAAAACTGAGGA	*Salmonella sp.*	*inv* A	(Hoorfar, et al. 2000)
SEFA-1 SEFA-2	GCAGCGGTTACTATTGCAGC CTGTGACAGGGACATTTAGC G	*Salmonella sp.*	*sef* A	(Woodward, et al. 1996)
Sal-F Sal-R	GCGTTCTGAACCTTTGGTAAT AA CGTTCGGGCAATTCGTTA	*Salmonella sp.*	*inv* A	(Daum, et al. 2002)
SHIMAF SHIMAR	CGTGCTCTGGAAAACGGTGA G CGTGCTGTAATAGGAATATCT TCA	*Salmonella sp.*	*him* A	(Chen, et al. 1999)
Fim1A Fim2A	CCTTTCTCCATCGTCCTGAA TGGTGTTATCTGCCTGACC	*Salmonella sp.*	*fim* A	(Cohen, et al. 1996)
p139 p141N	GTGAAATTATCGCCACGTTCG GGCAA TCATCGCACCGTCAAAGGAA CCGTAA	*Salmonella sp.*	*inv* A	(Rahn, et al. 1992)

Table 2.2 Cycling parameters for all primer sets used for the identification of *Salmonella* with Quantitative Real Time PCR

Fim 1A and Fim 2A; Styinva-JHO-2-left and Styinva-JHO-2-right

	Step	ºC	Time
	Initial denature	95	15 min
40x	Denature	95	30 sec
	Anneal	55	1 min
	Extend	72	30 sec

InvA-1F and InvA-1R; Sal-F and Sal-R

	Step	ºC	Time
	Initial denature	95	15 min
40x	Denature	95	30 sec
	Anneal	60	1 min
	Extend	72	30 sec

p139 and p141N

	Step	ºC	Time
	Initial denature	95	15 min
40x	Denature	95	15 sec
	Anneal	60	30 sec
	Extend	72	30 sec

SEFA-1 and SEFA-2

	Step	ºC	Time
	Initial denature	95	10 min
40x	Denature	95	1 min
	Anneal	55	1 min
	Extend	72	1 min

SHIMAF and SHIMAR

	Step	ºC	Time
	Initial denature	96	10 min
40x	Denature	95	1 min
	Anneal	57	1 min
	Extend	72	1 min

Table 2.3 MPN results from *Salmonella* spiked samples (TNTC = Too numerous to calculate) in bovine feces, porcine feces, carcass swab and ground beef samples and Spearman's rank correlation coefficient compared between the pre and post spiked samples

Known inoculated concentration CFU/mL	Bovine feces		Porcine feces		Carcass swab		Ground beef	
	Experimental result CFU/g	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)	Experimental result CFU/cm^2	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)
1.00E+01	0.0892		1.466		1.803		0.305	
1.00E+02	0.5756		23.027		0.761		4237.783	
1.00E+03	0.0892		91.783		4.760		10.846	
1.00E+04	TNTC	0.6988	919.070	0.9759	4.85E+05	0.6587	1.275	-0.0952
1.00E+05	1.58E+02	(0.0538)	1.69E+05	(0.0001)	TNTC	(0.0757)	0.690	(0.8225)
1.00E+06	1.96E+03		TNTC		TNTC		TNTC	
1.00E+07	3.31E+01		TNTC		1.27E+06		0.642	
1.00E+08	TNTC		TNTC		284.053		0.496	

Table 2.4 Q-PCR results from *Salmonella* spiked bovine feces, porcine feces, carcass swab and ground beef samples and Spearman's rank correlation coefficient compared between the pre and post spiked samples

Known initial concentration CFU/mL	Bovine feces		Porcine feces		Carcass swab		Meat	
	Experimental result CFU/g	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)	Experimental result CFU/cm^2	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)
1.00E+01	1.70E+01		0.00E+00		6.79E+03		5.40E+01	
1.00E+02	1.60E+01		0.00E+00		7.71E+03		1.61E+02	
1.00E+03	6.60E+01		3.93E+02		7.16E+03		7.40E+01	
1.00E+04	6.10E+01	0.9524	3.80E+03	0.994	7.76E+04	0.9762	1.85E+02	0.9762
1.00E+05	2.09E+02	(0.0003)	1.62E+04	(0.0001)	2.26E+05	(0.0001)	2.91E+03	(0.0001)
1.00E+06	9.00E+02		4.74E+05		6.41E+06		2.52E+04	
1.00E+07	3.44E+03		3.04E+06		8.32E+07		3.75E+05	
1.00E+08	3.18E+04		3.15E+07		1.46E+08		7.31E+05	

Table 2.5 Direct Dilution results from *Campylobacter coli* and *Campylobacter jejuni* spiked bovine feces and porcine feces samples and Spearman's rank correlation coefficient compared between the pre and post spiked samples

Known concentration CFU/mL	Bovine feces *C. coli*		Bovine feces *C. jejuni*		Porcine feces *C. coli*		Porcine feces *C. jejuni*	
	Experimental result CFU/g	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)
1.00E+01	0.00E+00		5.00E+05		2.91E+07		1.35E+07	
1.00E+02	6.28E+04		1.22E+06		3.77E+05		6.70E+04	
1.00E+03	1.41E+05		6.60E+04		9.26E+04		2.30E+05	
1.00E+04	3.98E+04	0.9286	2.19E+05	0.6429	3.22E+05	0.5714	2.97E+05	0.3095
1.00E+05	4.45E+05	(0.0009)	4.72E+05	(0.0856)	4.71E+05	(0.1390)	6.97E+04	(0.4556)
1.00E+06	2.58E+06		6.36E+06		4.15E+06		1.47E+05	
1.00E+07	5.85E+07		5.33E+07		5.96E+07		1.28E+07	
1.00E+08	4.72E+08		7.22E+08		1.69E+08		1.93E+08	

Table 2.6 MPN results from *Campylobacter coli* and *Campylobacter jejuni* spiked hide swab, carcass swab and ground beef samples and Spearman's rank correlation coefficient compared between the pre and post spiked samples (TNTC = Too numerous to count)

	Hide swab *C. jejuni*		Carcass swab *C. jejuni*		Ground beef *C. jejuni*		Hide swab *C. coli*		Carcass swab *C. coli*		Ground beef *C. coli*	
Known concentration CFU/mL	Experimental result CFU/cm^2	Spearman's R (P-value)	Experimental result CFU/cm^2	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)	Experimental result CFU/cm^2	Spearman's R (P-value)	Experimental result CFU/cm^2	Spearman's R (P-value)	Experimental result CFU/g	Spearman's R (P-value)
10	0.108	0.8721 (0.0539)	14.14	0.9411 (0.0051)	0	0.9411 (0.0051)	0	0.7826 (0.1176)	37.28	0.9258 (0.0080)	0	0.9258 (0.0080)
100	0		142.43		0		0		77.05		0.361	
1000	0.452		311		0.361		0		77.05		17.082	
10000	0.452		TNTC		0.361		46.2		TNTC		TNTC	
100000	TNTC		TNTC		0.361		9.32		TNTC		TNTC	
1000000	NA		TNTC		931.544		NA		TNTC		TNTC	

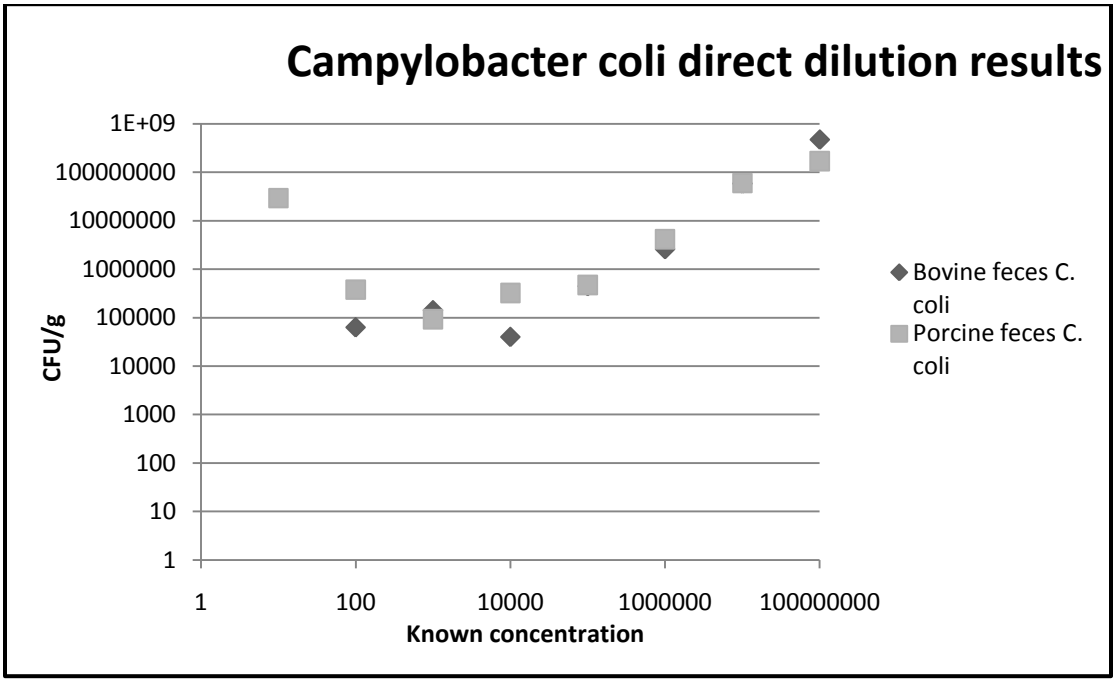

Figure 2.1 *Campylobacter coli* direct dilution in bovine and porcine feces results compared to the initial known starting concentration

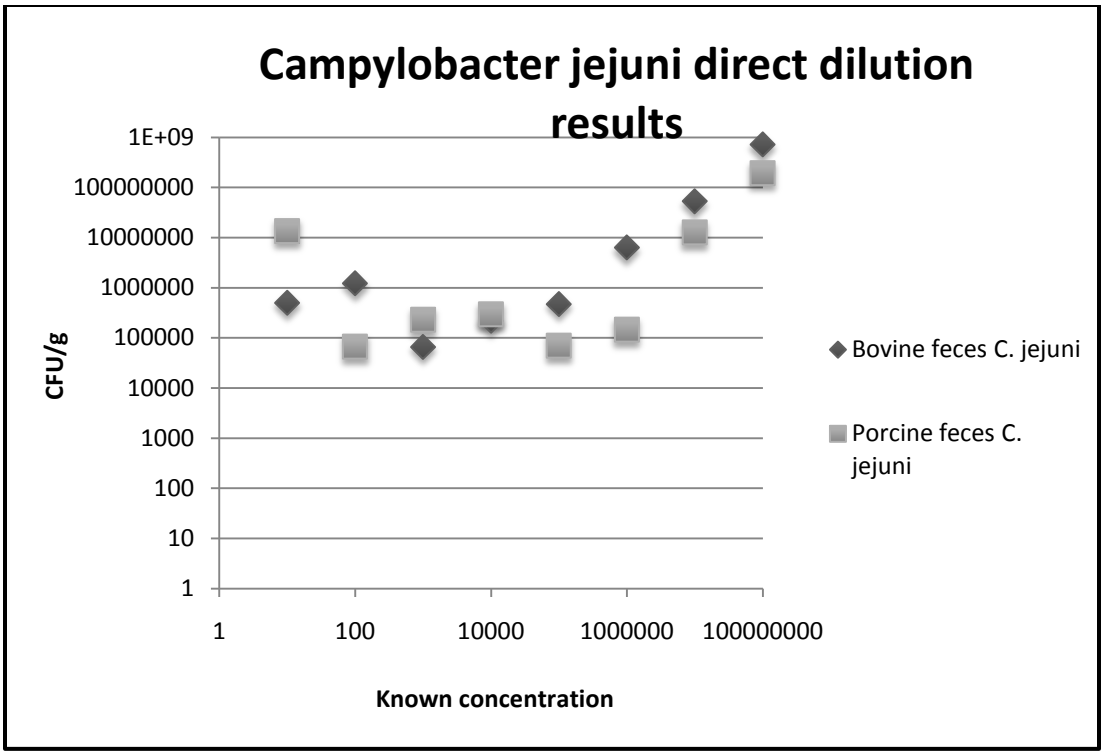

Figure 2.2 *Campylobacter jejuni* direct dilution in bovine and porcine feces results compared to the initial known starting concentration

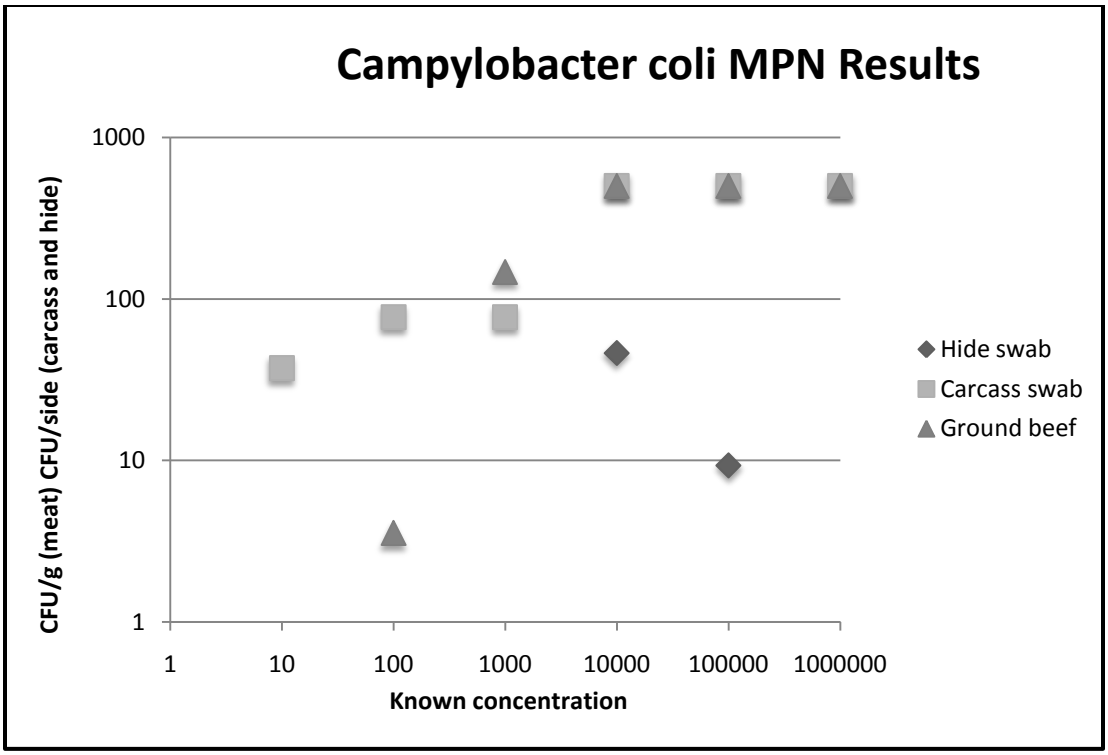

Figure 2.3 *Campylobacter coli* MPN hide, carcass and meat results compared to the initial known starting concentration

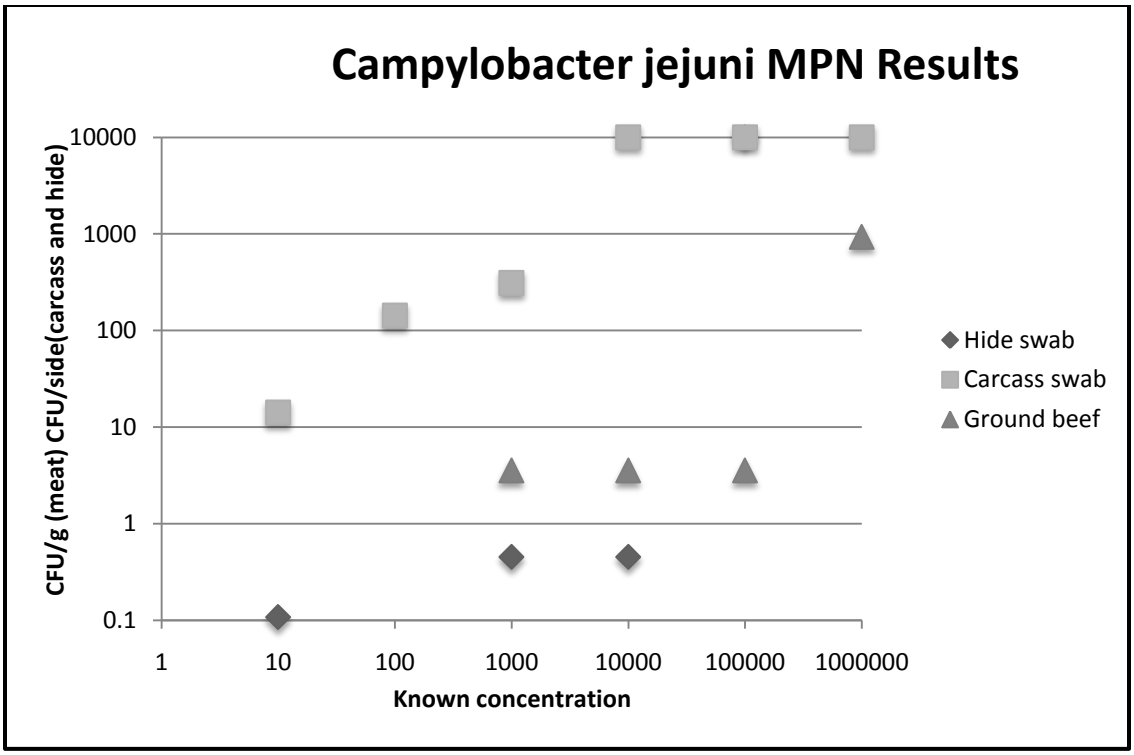

Figure 2.4 *Campylobacter jejuni* MPN hide, carcass and meat results compared to the initial known starting concentration

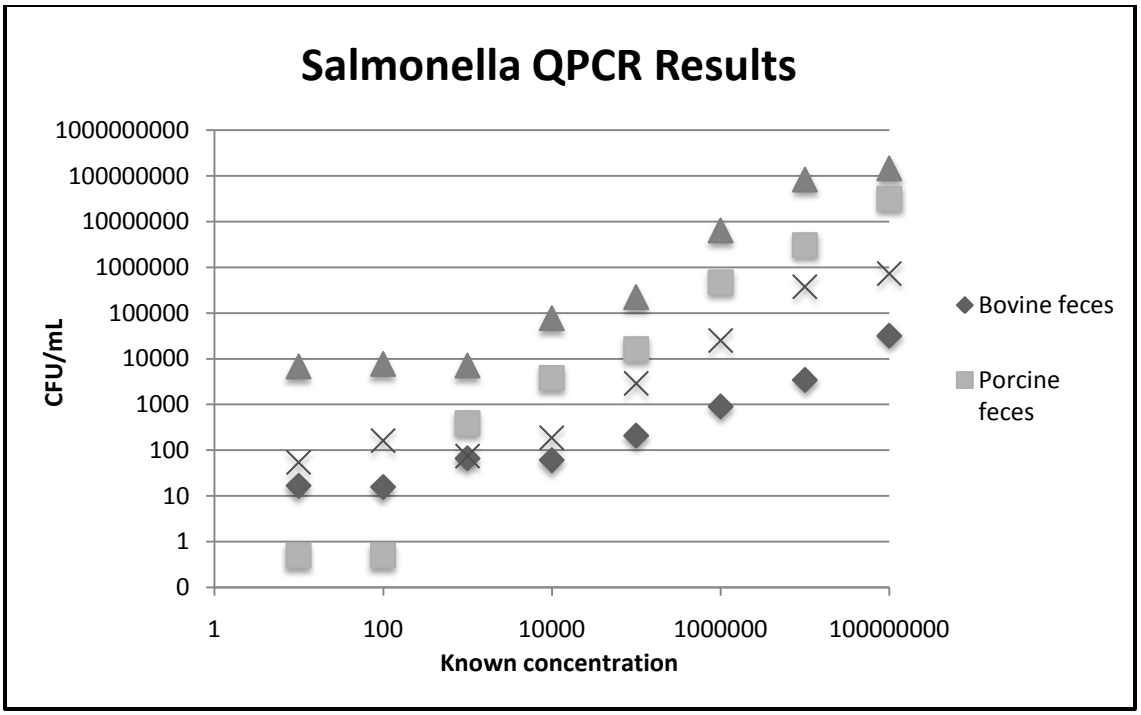

Figure 2.5 *Salmonella* QPCR results for all sample types compared to the initial known starting concentration

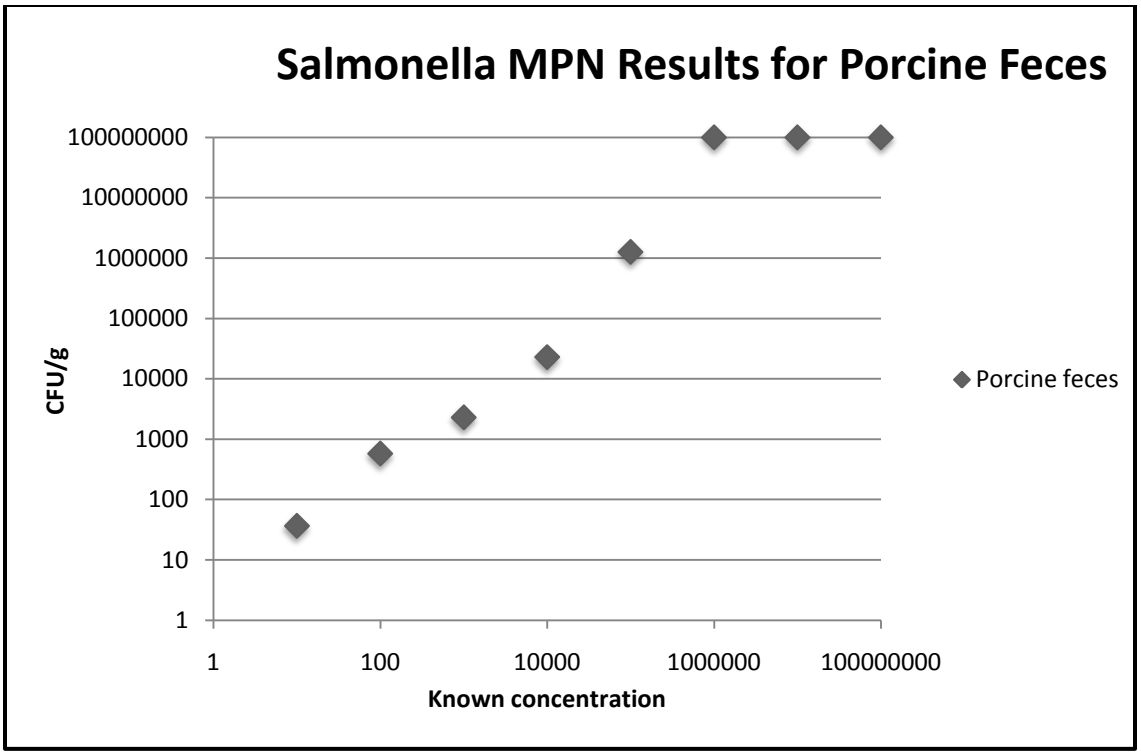

Figure 2.6 *Salmonella* MPN results in porcine feces compared to the initial known starting concentration

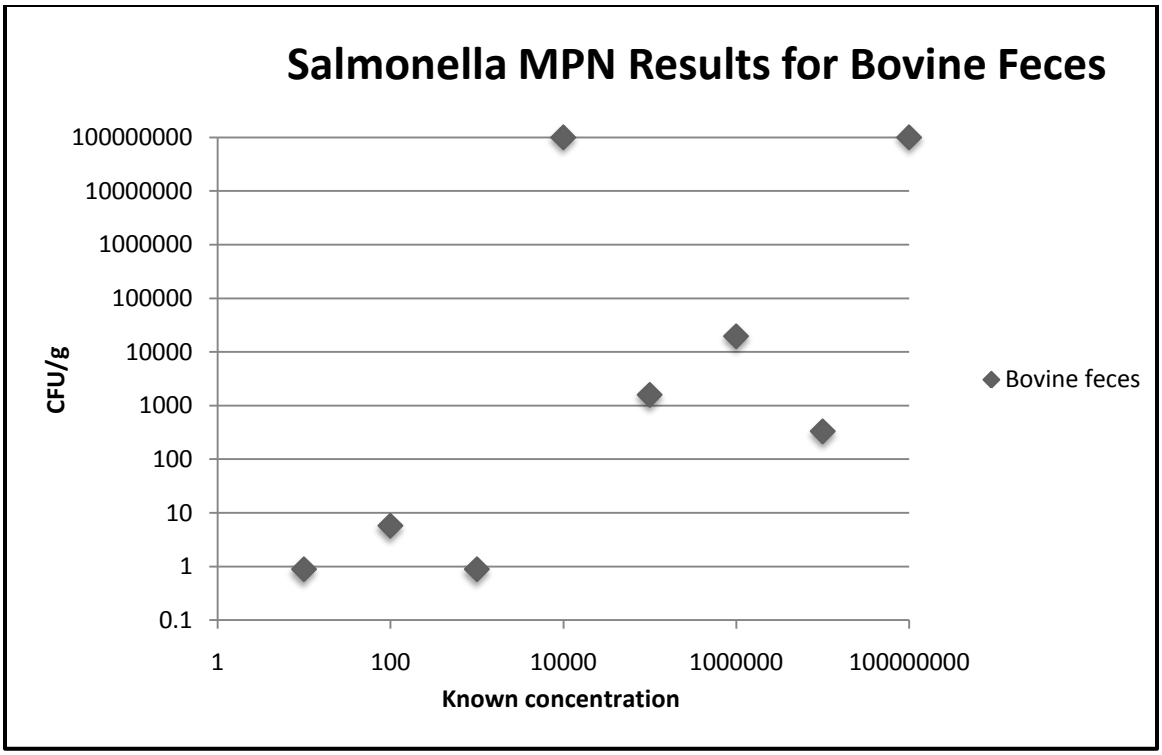

Figure 2.7 *Salmonella* MPN results in bovine feces compared to the initial known starting concentration

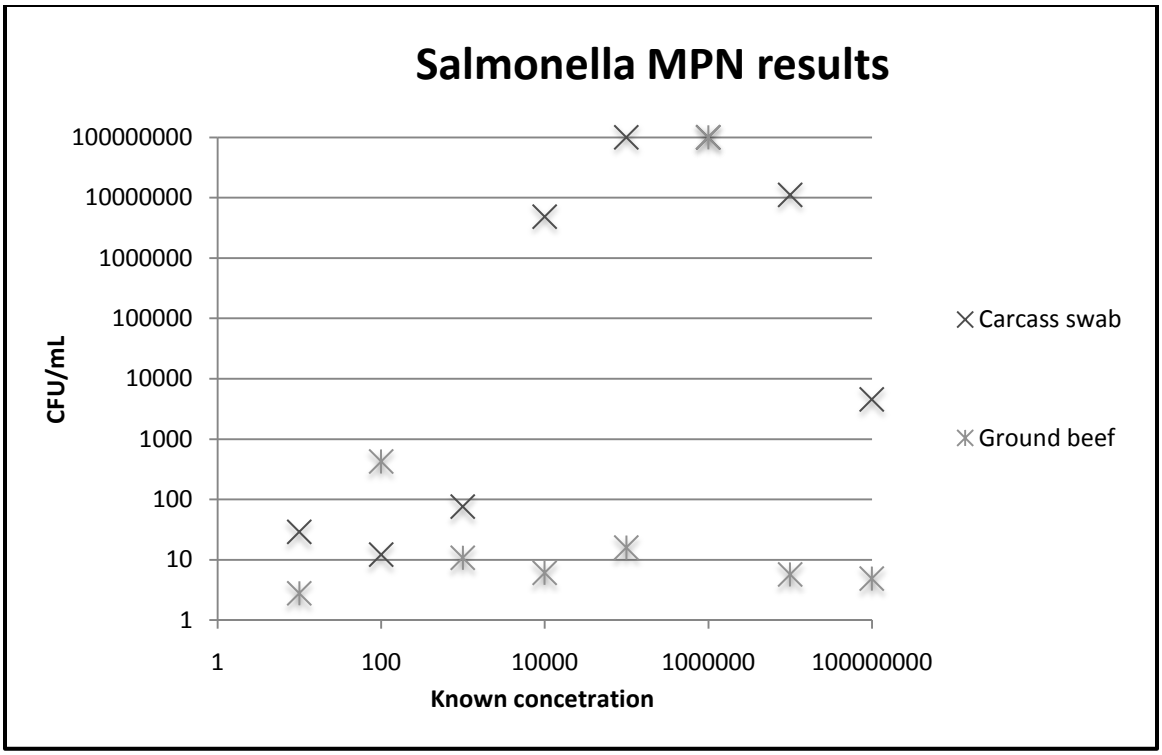

Figure 2.8 *Salmonella* MPN results for carcass and ground beef compared to the initial known starting concentration

Chapter 3 Quantification of *Campylobacter* in cattle before, during and after the slaughter process [1]

3.1 Abstract

Salmonella and *Campylobacter* cause a significant number of illnesses annually in the United States and around the world, most of which are food-related. Cattle can be asymptomatic carriers of both of these pathogens. The objective of this study was to determine the association between the concentration of *Salmonella* and *Campylobacter* pre- and post-harvest in cattle. Samples were collected from each of 98 individually identified cattle during the peri-harvest and post-harvest period. For each animal, four different phases were sampled: on farm (fecal sample), post-stunning and exsanguination (hide sponge and pre-fecal sample (lairage)), pre-chilling (carcass sponge) and final product (ground meat). *Salmonella* and *Campylobacter* were cultured and quantified at each stage by using the direct dilution and most probable number method. *Salmonella* was not isolated from any sample. The proportion (%) of samples that were *Campylobacter* positive was 77, 82, 97, 55, and 12 for farm, lairage, hide, carcass and meat samples respectively. The mean *Campylobacter* concentration for each sample was: fecal sample from farm, 3.7×10^4 cfu/g; pre-fecal sample from lairage, 1.6×10^5 cfu/g; hide sponge, 0.9 cfu/cm^2; carcass sponge 8.7 cfu/half carcass; and meat 1.1 cfu/g. There

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were no associations between *Campylobacter* concentrations for any two sample types. This lack of association could indicate that there is an environmental reservoir that can contaminate the final meat product, or since the majority of animals were positive entering the slaughter process, that the process itself reduces the load of *Campylobacter* regardless of the initial concentration. In addition, contamination of meat may be more strongly associated with peri-harvest practices than animal carriage rates.

3.2 Introduction

Salmonella and *Campylobacter* cause a significant number of illnesses annually in the United States and around the world, and most of these illnesses are food-related (Scallan, et al. 2011). *Campylobacter* has been isolated from healthy as well as diarrheic animals leading to fecal contamination of meat during processing, posing a food safety risk (Blaser, et al. 1980, Munroe, et al. 1983, Prescott, et al. 1981). Quantitative measures of food safety risk are rarely reported and represent a critical data gap for development of quantitative risk assessments. The objective of this study was to determine the association between the concentration of *Salmonella* and *Campylobacter* in bovine samples collected before and during the slaughter process with concentrations on meat. Our hypothesis was that there was a positive association between pathogen concentrations from samples taken during the harvest process and concentrations on meat.

3.3 Methods

3.3.1 Animals

A convenience sample of 98 steers was utilized for this project. All of the steers were reared at The Ohio State University owned farm located in Wooster, OH. The steers were shipped to two different slaughter facilities. One slaughter facility was privately owned (plant A) and the other was owned and run by The Ohio State University (OSU, plant B). All of the steers shipped to the OSU facility were sampled (N=38), but only the first ten steers slaughtered on each of six sampling days were sampled at the privately owned slaughter facility (N=60). Plant A processed the steers on six separate dates (April 3, 2006 N=10, April 10, 2006 N=10, April 17, 2006 N=10, April 24, 2006 N=10, May 1, 2006 N=10 and May 8, 2006 N=10). Plant B processed the steers on six separate dates as well (April 4, 2006 N=4, April 11, 2006 N=10, April 18, 2006 N=8, April 25, 2006 N=4, May 2, 2006 N=8 and May 9, 2006 N=4).

3.3.2 Sample Collection

Within 48 hours of transport to the slaughter facility, a fresh fecal sample (~20 g) was obtained from individually identified steers. A pre-fecal sample (~20 g) also was obtained from the rectum of the individually identified steers immediately post mortem. The area of the hide that was the most visually contaminated with feces and dirt was sampled immediately post mortem in three different areas of ~910cm^2 with sponges (hydrated-sponge, 3M, Saint Paul, MN). The entire hot carcass was sampled post-washing and pre-chilling with five sponges (3M) for each half of the carcass. The carcass was sampled by visually dividing the carcass into five areas (three sections on the outside and two sections on the inside), the outside was sampled first with three sponges, and then the inside was sampled. The following week after the slaughter of each group of

steers, an individual ground meat sample from each steer was obtained (~1 pound). All of the sponges were sterile and pre-moistened in 10 ml of buffered peptone water (BPW).

3.3.3. *Salmonella* MPN

The fecal and pre- fecal samples from farm and lairage were processed by adding 4 g of feces to 36 ml of tetrathionate broth (TTB, Becton Dickenson, Sparks, MD) in a three tube-four serial 10-fold dilution MPN method. The dilutions were conducted by taking 4 ml from the 10^{-1} dilution, which was then mixed in the next tube with 36 ml of TTB to make the 10^{-2} dilution and was continued to a final dilution of 10^{-4}. The fecal and pre-fecal samples from farm and lairage were incubated at 37° C for 24h. A 100µl aliquot from each tube was plated onto *Xylose* Lactose Tergitol 4 (XLT4, Becton Dickenson) agar plates and incubated for another 18 – 24h at 37° *C.* Plates were categorized yes/no with a yes indicating a black colony on XLT4.

The hide samples were processed by mixing the sponge with 30 ml of TTB. Ten milliliters of the mixture was added to three tubes and one ml was taken from the 10^{-1} dilution and added to 9 ml of TTB to make the next dilution and so on for the 3 tube x 4 dilution MPN. The hide samples followed the same protocol as the fecal samples from this point forward. The five sponges from the carcass were pooled and mixed with 150 ml of BPW. A 30 ml aliquot of the pooled carcass sample was added to each of three tubes and 3ml was added to 27 ml to make a 3 tube x 4 dilution MPN. The tubes were incubated for 24h at 37° *C.* One-hundred microliters from each tube was then added to 10 ml of Rappaport-Vassiliadis R10 (RV, Becton Dickenson) broth and incubated at 42° C for 18 – 24h. An aliquot of 100 µl from each tube was plated onto XLT4 agar plates and

incubated for another 18 – 24h at 37° *C.* Plates were read yes/no with a yes indicating a black colony on XLT4.

Ten grams of meat was mixed with 90 ml of BPW and 10 ml of this mixture was added to 90 ml of BPW to make the 3 tube, 4 dilution MPN. The samples followed the protocol for the carcass sponges from this point on.

3.3.4 *Campylobacter* direct dilution and MPN

The fecal and pre-fecal samples from farm and lairage were processed by adding 1 g of feces to 9 ml of BPW. Three dilutions were made taking 1 ml from the initial 10^{-1} dilution and adding to 9 ml of BPW, etc to a maximum dilution of 10^{-4}. One-hundred microliters from each dilution was plated in duplicate on Campy-Cefex ((Oyarzabal, et al. 2005)) plates and incubated under microaerophilic conditions (O_2 5-15% and CO_2 5-12%) for 48h at 42° *C. Campylobacter* suspect colonies were counted.

The hide samples were processed by mixing the sponge with 30 ml of Bolton broth (Oxoid, Hampshire, United Kingdom). Ten milliliters of the mixture were added to three tubes and one milliliter was taken from the 10^{-1} dilution and added to 9 ml of Bolton broth to make the next dilution and so on for the 3 tube x 4 dilution MPN. The tubes were incubated under microaerophilic conditions for 48h at 42° *C.* One-hundred microliters from each dilution was plated onto Campy-Cefex plates and incubated under microaerophilic conditions for 48h at 42° *C.* The plates were read yes/no for *Campylobacter* suspect colonies.

The five sponges from the carcass were pooled and mixed with 90 ml of Bolton broth. A 30 ml aliquot of the pooled carcass sample was added to each of three tubes and

3 ml from the 10^{-1} dilution was added to 27 ml of Bolton broth to make a 3 tube x 4 dilution MPN. The samples then followed the protocol for the hide sponges.

Ten grams of ground meat was mixed with 90 ml of Bolton broth and 10 ml of this mixture was added to 90 ml of Bolton broth to make the 3 tube x 4 dilution MPN. The samples then followed the protocol for the hide and carcass sponges. Suspect *Campylobacter* colonies were stored at -80° C in Brucella broth (Becton Dickenson) and 10% glycerol for further processing. The MPN was calculated based on the spreadsheet and recommendations by Garthright and Blodgett (Garthright, et al. 2003).

3.3.5 *Campylobacter* confirmation and speciation

After all the samples were processed, the *Campylobacter* suspect colonies were revived from the freezer onto Mueller Hinton (MH, Becton Dickinson) plates. Catalase (Becton Dickenson) and oxidase (Becton Dickenson) tests were performed and all colonies that were positive for both in addition to colonies that were positive for oxidase only were grown in brucella (Becton Dickenson) broth for 72h at 42° C for DNA extraction using the DNeasy Tissue kit (Qiagen, Valencia, CA). PCR was performed on the extracted DNA targeting the *hipO* gene for *Campylobacter jejuni* and the *glyA* gene for *Campylobacter coli* ((LaGier, et al. 2004)).

3.3.6 Statistical analysis

Descriptive statistics were performed for prevalence, mean and median concentration. Spearman's Rank Correlation Coefficient was calculated to ascertain correlations of *Campylobacter* concentrations between meat samples and each other sample type (feces from farm and lairage, hide and carcass). Odds ratios were calculated to determine the odds of a meat sample being positive when fecal samples from farm,

fecal samples from lairage, hide sponge or carcass sponge samples were positive. All statistics were performed with the STATA statistical software (Intercooled STATA 9, StataCorp, College Station, TX).

3.4 Results

3.4.1 Prevalence of *Salmonella*

There were no *Salmonella* isolated from any sample.

3.4.2 Prevalence and MPN concentrations for *Campylobacter*

The proportion (%) of samples that were *Campylobacter* positive was 77, 82, 97, 55, and 12 for fecal samples from farm, pre-fecal samples from lairage, hide sponges, carcass sponges and meat samples, respectively (Fig. 1). The mean *Campylobacter* concentration for each sample type was: fecal sample from farm, 3.7×10^4 cfu/g; pre-fecal sample from lairage, 1.6×10^5 cfu/g; hide sponge, 0.9 cfu/cm^2; carcass sponge 8.7 cfu/half carcass; and meat 1.1 cfu/g (Fig. 2). The median *Campylobacter* concentration for each sample type was farm, 3.0×10^3 cfu/g; lairage, 9.8×10^3 cfu/g; hide sponge, 1.2×10^2 cfu/cm^2; carcass sponge, 1.2×10^3 cfu/half carcass; and meat, 0 cfu/g.

3.4.3 Speciation of *Campylobacter*

Of the 351 putative *Campylobacter* isolates, 242 (69%) were catalase and oxidase positive, 80 (23%) were negative for oxidase, and 29 (8%) were not recovered from cryopreservation. Of the 242 isolates with biochemical reactions consistent with *Campylobacter*, 93 (38%) were *C. jejuni*, 88 (36%) were not *C. jejuni* or *C. coli*, 71 (29%) were *C. coli*, and 18 (7%) were positive for both *C. jejuni* and *C. coli*. There was a

shift from predominantly *C. jejuni* recovered from the fecal samples to only *C. coli* recovered from the meat samples (Fig. 3).

3.4.4 Statistical analysis

There was no correlation between the concentration of *Campylobacter* in "upstream" samples and concentration in meat samples (Table 3.1). The univariate analysis of odds ratios (Table 3.2) found that *Campylobacter* contamination of the hide was associated with a lower odds of *Campylobacter* contamination in ground beef (OR=0.06, P=0.04). No other associations were identified.

3.5 Discussion

There have been other reports of *Salmonella* isolated from cattle feces at prevalence rates ranging from 0.08% to 46% (Van Donkersgoed, et al. 1999, Barham, et al. 2002, Fedorka-Cray, et al. 1998, Beach, et al. 2002). The relatively small sample size of our study coupled with the cattle all being housed on the same farm may have contributed to our inability to isolate *Salmonella*.

Campylobacter prevalence on farms around the world can vary widely, ranging from 0.8% to 84% (Rosef, et al. 1983)(Hoar, et al. 2001);(Cabrita, et al. 1992); (Bolton, et al. 1982); (Hakkinen, et al. 2007); (Giacoboni, et al. 1993); (Pezzotti, et al. 2003); (Bailey, et al. 2003)(Oporto, et al. 2007); (Inglis, et al. 2003).

The prevalence of *Campylobacter* on cattle hides we observed in this study differed considerably from a previous report where *Campylobacter* was not recovered (Reid, et al. 2002). However, we sampled an area of the hide that was about nine times larger than they sampled, and we chose to sample the area on the hide which appeared to

have the highest contamination of dirt and feces. The previous study ranked their cattle as being "visually clean" meaning there was no visible mud/feces on the hides and the hides were dry, which was not what we observed for the cattle in our study.

We found a higher proportion of *Campylobacter* positive carcasses than has been reported in a number of previous studies (Hakkinen, et al. 2007, Christensen, et al. 1994, Madden, et al. 2001, McNamara. 1995, Vanderlinde, et al. 1998) . One explanation is that we sampled more surface area, i.e. an entire half carcass, than had been sampled previously. This difference in sampling technique may have contributed to the difference in prevalence, with our increased sampling effort resulting in increased sensitivity for detection of *Campylobacter*. We also made multiple dilutions of each sample that may have increased the sensitivity of culturing *Campylobacter*.

The prevalence of *Campylobacter* in the ground beef that we observed is comparable to other studies that reported prevalence ranging from 0 to 23.6% in various types of retail beef products ((Pezzotti, et al. 2003, Ghafir, et al. 2007, Hong, et al. 2007, Madden. 2002, Ono, et al. 1999, Taremi, et al. 2006, Whyte, et al. 2004, Wong, et al. 2007, Zhao, et al. 2001, Cloak, et al. 2001, Duffy, et al. 2001, Fricker, et al. 1989, Korsak, et al. 1998, Osano, et al. 1999)).

The shift in *Campylobacter* species between feces (90% *C. jejuni*) to meat (100% *C. coli*) was unexpected and may represent a natural shift in the predominant *Campylobacter* species present in these different environments. Another possible explanation for this observed change may be that the culture methods we used for the fecal samples and the hide, carcass and meat differed substantially. The fecal samples were direct-plated and were not enriched in Bolton broth prior to plating, as were the hide

sponge, carcass sponge and ground beef. According to the manufacturers of the different media that we used, Bolton broth is recommended for all thermo-tolerant *Campylobacter*, and Campy-cefex is recommended for *C. jejuni*, *C. coli*, and *C. lari.* We found no reports of Campy-cefex or Bolton broth preferentially selecting for one species over another. Thus, it is unclear if the media we used played a role in the species of *Campylobacter* we recovered. Further investigations comparing different culture methods and *Campylobacter* species selection may help to clarify this issue.

One limitation of this study is that 80 isolates (23%) were negative for oxidase and were therefore not considered to be *Campylobacter*. Since the confirmation was completed after recovery following regrowth from freezer stock, the *Campylobacter* might not have survived and a contaminant was evaluated instead.

We also had 88 putative *Campylobacter* isolates (36%) that were neither *C. jejuni* nor *C. coli* based on PCR of the *hipO* gene or *glyA* gene. Other studies have identified *C. fetus* subsp. f*etus, C. lanienae, C. hyointestinalis* in addition to *C. jejuni* and *C. coli* in cattle (Hakkinen, et al. 2007, Pezzotti, et al. 2003, Inglis, et al. 2003, Manser, et al. 1985). The unidentified isolates could be another species of *Campylobacter* or another organism that is oxidase positive and can tolerate the antibiotics and selective pressure of the Campy-cefex plate. These isolates were scattered throughout the different sample types and there was no obvious clustering.

Relatively few studies have quantified *Campylobacter* in cattle. A study of beef cattle going to slaughter found an average MPN per g of 6.1×10^2 in pre-fecal material from the small intestine (Stanley, et al. 1998). In the United States a study was conducted that found a mean *C. jejuni* concentration of 0.1 MPN/cm^2 on carcasses (McNamara.

1995). In studies analyzing meat contamination, one reported that only one of four minced beef samples contained *C. jejuni* above the detection level of 5 to 10 MPN per g (Cloak, et al. 2001). Another study found 8/230 samples of beef were contaminated at a level of 0.3 MPN per g (Wong, et al. 2007). Other studies quantifying *Campylobacter* on the carcass and in the meat are consistent with our results (McNamara. 1995) .

The univariate analysis of odds ratios found a protective effect of contamination of the hide on the odds of contamination on the retail meat product. This result is biologically difficult to explain. One possibility for this relationship is that almost all samples were positive on the hide so the high number of positives may have impacted the statistical interpretation. We believe that this result is not biologically significant, because the idea that a contaminated hide leads to less contamination of the meat is not a biologically plausible explanation.

Ingestion of only 500 cells of *Campylobacter* can lead to clinical illness in humans (Black, et al. 1988, Deming, et al. 1987, Robinson, et al. 1979). Such a low infectious dose shows the importance of reducing the prevalence of *Campylobacter* in retail beef samples. Based on the average concentration from this study an individual need only consume 500 g of raw meat to receive an infectious dose. It also is possible for cross-contamination though the transfer of *Campylobacter* cells from the raw meat to other surfaces in the kitchen, which could then lead to infection.

3.6 Conclusions

The overall objective of this study was to determine if there was any association with the final meat product and the other samples collected "upstream." No statistically

significant associations were found. One explanation is that there are no associations between the concentration or prevalence of *Campylobacter* in the feces, on the hide or carcass and the final retail meat product. This explanation implies that there is an environmental reservoir that contaminates the final meat product, or since the majority of animals were positive entering the slaughter process, that the process itself reduces the load of *Campylobacter* regardless of the initial concentration. Contamination of the meat might also be more influenced by peri-harvest practices than it is by animal carriage rates. These associations could be confounded by the shift in species from *C. jejuni* to *C. coli*. The different culture methods also could account for the lack of association, by having different selection pressures and allowing different sets of *Campylobacter* to be cultured. The individual animal also might not be the best level to look at these associations. Instead it might be more appropriate to look at a group or herd level.

3.7 Acknowledgments

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Table 3.1 Association between concentration of *Campylobacter* in meat compared to farm, lairage, hide and carcass samples from cattle. (Spearman's rank correlation coefficient)

	Spearman's R	P-value
Fecal sample from farm	-0.008	0.94
Pre-fecal sample from lairage	0.0092	0.93
Hide sponge	-0.1367	0.19
Carcass sponge	-0.0456	0.66

Table 3.2 Univariate odds Ratios for the risk of *Campylobacter* positive meat sample with the following exposures (farm positive, lairage positive, hide positive or carcass positive) from cattle.

Risk for *Campylobacter* positive meat samples			
Exposure	OR	95% CI	P-value
Pre-fecal sample from farm	3.08	0.37-25.5	0.45
Fecal sample from lairage	2.6	0.34-20.65	0.69
Hide sponge	0.06	0.005-0.71	0.04
Carcass sponge	0.77	0.23-2.59	0.76

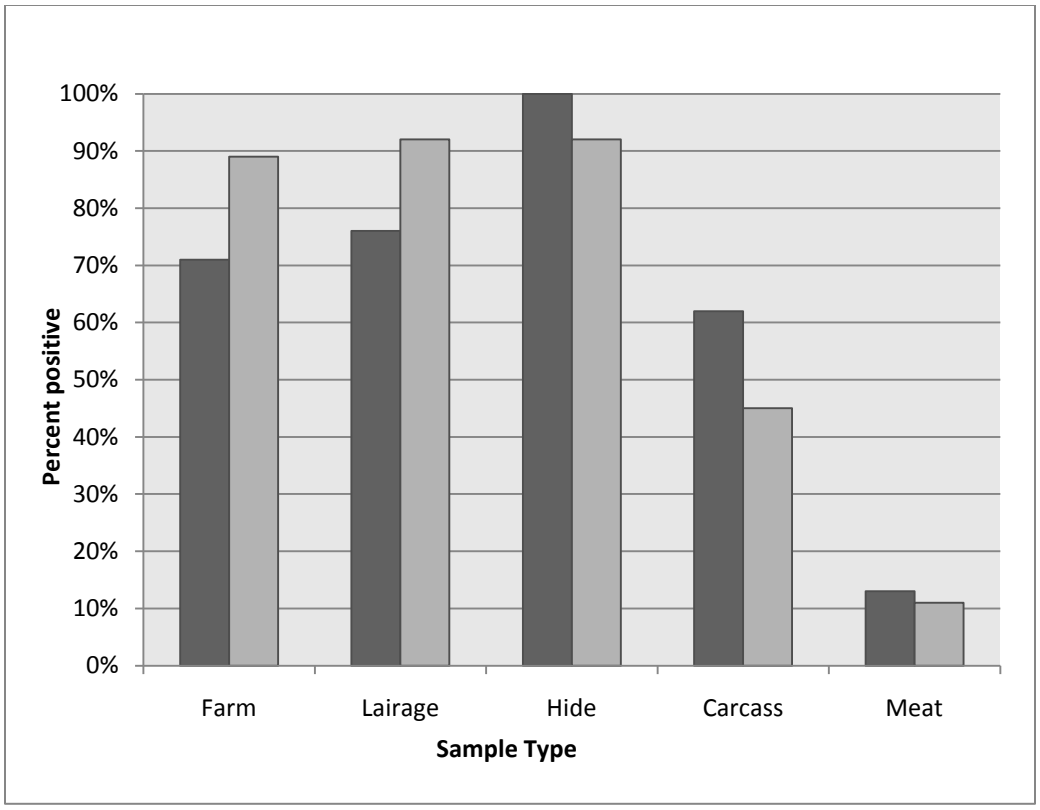

Figure 3.1 The prevalence of *Campylobacter* sp. isolated from the different sample types (farm, lairage, hide, carcass and meat) in cattle N=98. The dark grey bars represent plant A and the light grey bars represent plant B.

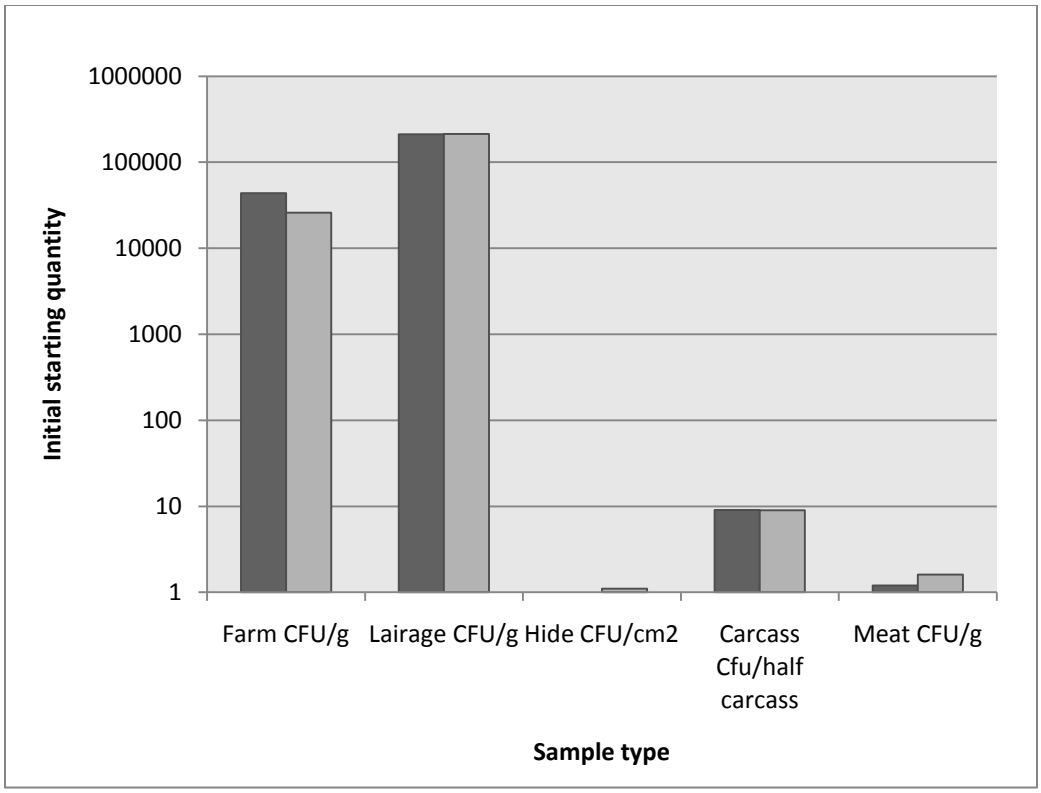

Figure 3.2 The mean value of the quantitative count of *Campylobacter* sp. from all samples (farm, lairge, hide, carcass, and meat) using direct dilution for farm and lairage and MPN for hide, carcass and meat from cattle. The dark grey bars represent plant A and the light grey bars represent plant B.

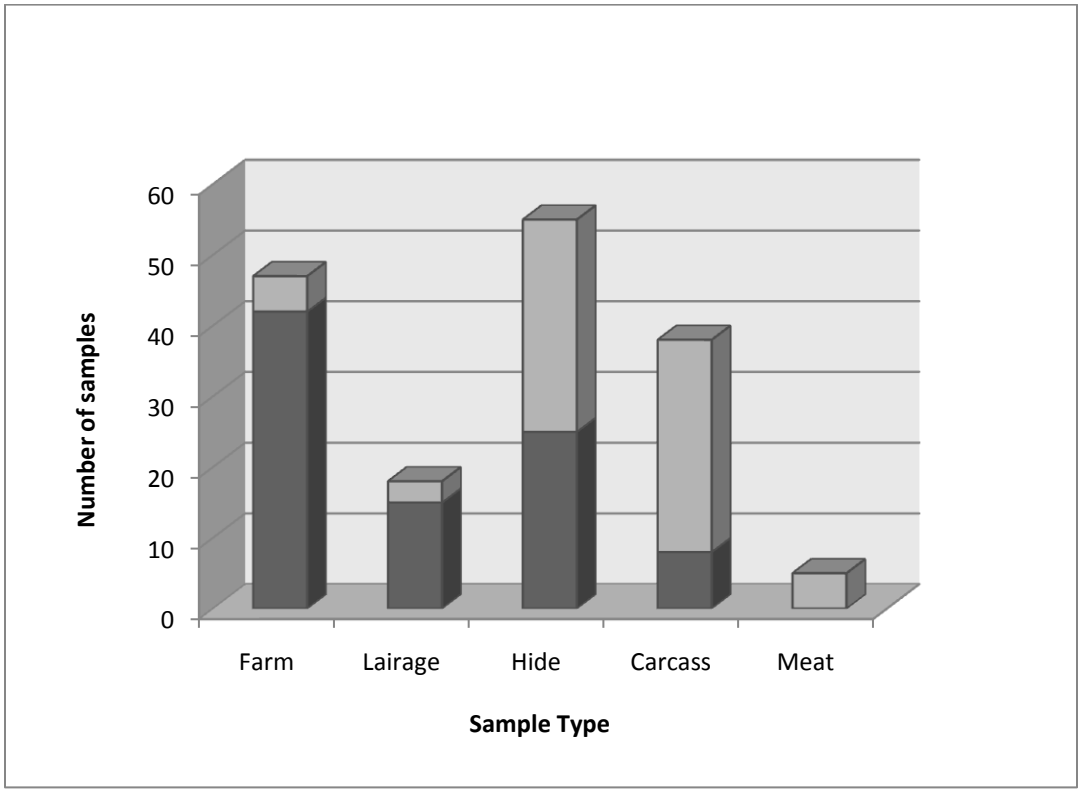

Figure 3.3 Species results of *Campylobacter* determined by PCR (hipO – *C. jejuni*, glyA – *C. coli*) for all sample types (farm, lairgae, hide, carcass, and meat) from cattle. The light grey bars represent *C. coli* and the dark grey bars represent *C. jejuni*.

Chapter 4 Quantification of *Campylobacter* in swine before, during and after the slaughter process[2]

4.1 Abstract

Salmonella and *Campylobacter* have been annually implicated in a large number of food-borne illnesses in the United States. The objective of this study was to determine the association between the concentration of *Salmonella* and *Campylobacter* peri- and post-harvest in swine. Samples were collected from each of 100 individually identified swine during the peri-harvest and post-harvest period. For each animal, four phases were sampled: on farm (fecal sample), in lairage (hide swab), post-stunning and exsanguination (pre-fecal sample (lairage)), pre-chilling (carcass swab). A final product (rib meat) sample from each animal was cultured for *Salmonella* and *Campylobacter* at each stage using direct dilution and the most probable number method. For the results, *Salmonella* was isolated from only two samples. The proportion (%) of samples that were *Campylobacter* positive was 90, 95, 76, 100, and 49 for farm, lairage, hide, carcass and meat samples respectively. The mean *Campylobacter* concentration for each sample

[2] Submitted to Foodborne Pathogens and Diseases authors: M. J. Abley, T. E. Wittum, S. J. Moeller, H. N. Zerby, and J. A. Funk

was: farm, 1.7 x 10^6 cfu/g; lairage, 1.2 x 10^7 cfu/g; hide swab, 1.4 cfu/cm^2; carcass swab, 1.7 x 10^3 cfu/half carcass; and ribs, 18 cfu/g. There was a positive correlation between *Campylobacter* concentrations in feces (farm, R = 0.20 [p=0.065] and concentration of *Campylobacter* on meat and between fecal concentration at lairage, R=0.20 [p=0.068]) and concentration in on meat, using the Spearman's R correlation. There was no association between the isolation of *Campylobacter* on meat and the isolation of *Campylobacter* at any peri-harvest stage. This could indicate that the more important predictor of the final meat product being contaminated is a pig that sheds higher concentrations of *Campylobacter* before slaughter. This study found a high concentration and prevalence of *Campylobacter* on samples taken peri- and post-harvest indicating a need for further investigations to try to limit the risk of *Campylobacter* contamination on pork products.

4.2 Introduction

Salmonella and *Campylobacter* are estimated to cause 1.8 million illnesses annually in the United States, and most of these illnesses are food-related (Scallan, et al. 2011). Pigs can be sub-clinically infected with these pathogens and fecal contamination of meat during slaughter is a food safety risk (Tam, et al. 2003, Frost. 2001, Kramer, et al. 2000, Gillespie, et al. 2002). For preharvest control, it is unclear whether intervention strategies should focus more on elimination of pathogens or decreasing the concentration of pathogens shed by animals, which has significant implications for both the cost and efficiency for pre-harvest control programs. Although *C. jejuni* is considered the most important *Campylobacter* species causing infection in humans, recent studies have highlighted the importance of *C. coli* (most common in swine) as an additional human

pathogen also causing foodborne illness, with an added challenge of being more frequently resistant to antimicrobials (Tam, et al. 2003, Gillespie, et al. 2002, Englen, et al. 2005, Sails, et al. 2003, Bywater, et al. 2004). The objective of this study was to determine the association between the concentration of *Salmonella* and *Campylobacter* in fecal, pre-fecal, hide, carcass and meat samples collected before or during the slaughter process with concentrations of these organisms, if observed, in pork. The hypothesis was that there would be a positive association between *Campylobacter* and *Salmonella* concentration and prevalence between samples taken before and during the harvest when compared with concentration or prevalence in a final meat product.

4.3 Methods

4.3.1 Animals

Samples from 100 pigs raised at The Ohio State University's Western Agricultural Research Station (South Charleston, OH) were utilized for the study. Pigs were raised in deep, straw-bedded finishing facilities with a solid concrete base and provided ad libitum access to feed and water throughout the finishing period. Pigs were were transported approximately 74 kg, placed in lairage, and slaughtered following an 18 h fast at the The Ohio State University Meat Science Laboratory. Pigs were rendered unconscious by electrical stun, exsanguinated, and placed in a scald tank (5 min @ 61.5° C), dehaired, flamed and rinsed prior to evisceration.

4.3.2 Sample collection

The date of harvest and the number of pigs harvested on each date are as follows, Oct. 12, 2006 (10), Nov. 2, 2006 (10), February 1, 2007 (16), February 8, 2007 (16),

February 15, 2007 (16), February 22, 2007 (16) and March 1, 2007 (16). Within 18 hours of transport to the slaughter facility, a fresh fecal sample (~20 g) was obtained from individually identified pigs. The area of the hide which was most visibly contaminated with mud and feces was also swabbed while the pigs were in lairage (in three different areas of ~625 cm^2 each with sponges). A pre-fecal sample (~20 g) also was obtained from the individually identified pigs immediately post mortem from the rectum. The entire hot carcass was swabbed following washing and prior to chilling using three sponges for each half of the carcass. One week after slaughter, ~1,500 g of ribs (meat and bone) was obtained from each carcass. All sponges were sterile and pre-moistened in 10ml of buffered peptone water (hydrated-sponge, 3M, Saint Paul, MN) prior to application. Samples were divided for *Salmonella* and *Campylobacter* culture as follows: sections of the fecal material were weighed for each organism, one of the three hide swabs was used for each culture, one half of each carcass was used for each organism and 500g of the 1,500 g obtained was used for each organism.

4.3.3 *Salmonella* MPN

Fecal and pre-fecal samples from farm and lairage were processed by adding 4 g of feces to 36 ml of Tetrathionate broth (TTB, Becton Dickenson, Sparks, MD) in a three tube-four serial 10-fold dilution MPN method. The dilutions were conducted by taking 4 ml from the 10^{-1} dilution, which was then mixed in the next tube with 36ml of TTB to make the 10-2 dilution and this, was continued to a final dilution of 10^{-4}. The fecal samples from farm and lairage were incubated at 37° C for 24 h. A 100 µl aliquot from each tube was plated onto *Xylose* Lactose Tergitol 4 (XLT4, Becton Dickenson) agar

plates and incubated for another 18 – 24 h at 37° *C.* Plates were read yes/no with a yes indicating a black colony on XLT4.

The hide swab samples were processed by mixing the sponge with 30 ml of TTB. Ten ml of the mixture was added to three tubes and one ml was taken from the 10^{-1} dilution and added to 9 ml of TTB to make the next dilution and repeated for the 3 tube x 4 dilution MPN. The hide swab samples followed the same protocol as the fecal samples from this point forward. The 3 sponges from the carcass swab were pooled and mixed with 150 ml of BPW. A 30 ml aliquot of the pooled carcass swab sample was added to each of three tubes and 3ml was added to 27 ml to make a 3 tube x 4 dilution MPN. The tubes were incubated for 24 h at 37° *C.* One-hundred microliters from each tube was then added to 10 ml of Rappaport-Vassiliadis R10 (RV, Becton Dickenson) broth and incubated at 42° C for 18 – 24 h. An aliquot of 100 µl from each tube was plated onto XLT4 agar plates and incubated for another 18 – 24 h at 37° *C.* Plates were read yes/no with a yes indicating a black colony on XLT4.

One pound (454 g) of meat was mixed with 500 ml of BPW and 10 ml of this mixture was added to 90 ml of BPW to make the 3 tube 4 dilution MPN. The samples then followed the protocol for the carcass swabs from this point on.

4.3.4 *Campylobacter* direct dilution and MPN

Fecal and pre-fecal samples from the farm and lairage were processed by adding 1g of feces to 9 ml of BPW. Three dilutions were made taking 1 ml from the initial 10^{-1} dilution and adding to 9 ml of BPW repeated a maximum dilution of 10^{-4}. One-hundred microliters from each dilution was plated in duplicate on Campy-Cefex (Oyarzabal, et al.

2005) plates and incubated under microaerophilic conditions for 48 h at 42° *C. Campylobacter* suspect colonies were then counted.

The hide swab samples were processed by mixing the sponge with 30 ml of Bolton broth (Oxoid, Hampshire, United Kingdom). Ten ml of the mixture was added to three tubes and one ml was taken from the 10^{-1} dilution and added to 9 ml of Bolton broth to make the next dilution and repeated to complete the 3 tube x 4 dilution MPN. The tubes were incubated under microaerophilic conditions for 48 h at 42° *C.* One-hundred microliters from each dilution was plated onto Campy-Cefex plates and incubated under microaerophilic conditions for 48 h at 42° *C.* The plates were read yes/no for *Campylobacter* suspect colonies.

The 3 sponges from the carcass swab were pooled and mixed with 90 ml of Bolton broth. A 30 ml aliquot of the pooled carcass swab sample was added to each of three tubes and 3ml from the 10^{-1} dilution was added to 27 ml of Bolton broth to make a 3 tube x 4 dilution MPN. The samples then followed the protocol for the hide swabs from this point on.

One pound (454 g) of meat was mixed with 500 ml of Bolton broth and 10 ml of this mixture was added to 90 ml of Bolton broth to make the 3 tube 4 dilution MPN. The samples then followed the protocol for the hide swab and carcass swabs from this point on. Suspect *Campylobacter* colonies were saved at -80° C in Brucella broth (Becton Dickenson) and 10% glycerol for further processing.

4.3.5 *Salmonella* confirmation

Black colonies on XLT4 plates were subcultured on nutrient agar slants and stored at 4° C until the all the samples were processed. The *Salmonella* suspect colonies were then struck onto MacConkey agar and the non-lactose fermented colonies were biochemically confirmed on urea and triple sugar iron slants (TSI, Becton Dickenson).

4.3.6 *Campylobacter* confirmation and speciation

After all the samples were processed the *Campylobacter* suspect colonies were revived from the freezer onto Mueller Hinton (MH, Becton Dickinson) plates. Catalase (Becton Dickenson) and oxidase (Becton Dickenson) tests were performed and all colonies that were positive for both in addition to colonies that were positive for oxidase only were grown in Brucella (Becton Dickenson) broth for 72 h at 42° C for DNA extraction using the DNeasy Tissue kit (Qiagen, Valencia, CA). PCR was performed on the extracted DNA targeting the *hipO* gene for *Campylobacter jejuni* and the *glyA* gene for *Campylobacter coli* (LaGier, et al. 2004).

4.3.7 Statistical analysis

Descriptive statistics were performed for prevalence, mean and median concentration of *Salmonella* and *Campylobacter* for each sample type. Spearman's Rank Correlation Coefficient was calculated to ascertain correlations between meat samples and 1) fecal samples from farm, 2) pre-fecal samples from lairage, 3) hide swab and 4) carcass swab samples. A Spearman's R is interpreted as a value of 0 indicating no association, > 0 to 0.5 indicating a weak positive correlation, >0.5 to <1 indicating a strong positive correlation and 1 a perfect positive correlation. Univariate odds ratios were calculated to determine the odds of a meat sample being positive when fecal samples from farm, pre-fecal samples from lairage, hide swab or carcass swab samples

were positive. All statistics were performed with the STATA statistical software (Intercooled STATA 9, StataCorp, College Station, TX).

4.4 Results

4.4.1 *Salmonella* prevalence

Only two samples one from the farm and one from lairage from two different pigs were *Salmonella* culture positive. Therefore, no further analysis of *Salmonella* results is reported.

4.4.2 Prevalence and MPN concentrations for *Campylobacter*

The proportion (%) of samples that were *Campylobacter* positive was 90, 95, 76, 100, and 49 for fecal samples from farm, pre-fecal samples from lairage, hide swabs, carcass swabs and rib samples, respectively (Fig. 1). The mean *Campylobacter* concentration for each sample type was farm, 1.7×10^6 cfu/g; lairage, 1.2×10^7 cfu/g; hide swab, 1.4 cfu/cm^2; carcass swab, 1.7×10^3 cfu/half carcass; and ribs, 18 cfu/g (Fig. 2). The median *Campylobacter* concentration for each sample type was farm, 1.2×10^5 cfu/g; lairage, 1.1×10^6 cfu/g; hide swab, 1.4×10^2 cfu/cm^2; carcass swab, 1.2×10^3 cfu/half carcass; and ribs, 1.4×10^2 cfu/g.

4.4.3 Speciation of *Campylobacter*

A total of 403 putative *Campylobacter* colonies were frozen and 309 (76.67%) samples were successfully recovered from the freezer. From the 309 samples a total of 301 (97.4%) were PCR confirmed as *C. coli* and one sample was also PCR positive for *C. jejuni*. The remaining eight samples were not further tested.

4.4.4 Statistical analysis

A weak positive correlation between *Campylobacter* concentrations in feces (fecal sample from farm, R= 0.20 [P = 0.065] and pre-fecal sample from lairage, R = 0.20 [P = 0.068]) and concentration of *Campylobacter* on ribs was found (Table 4.1). The univariate analysis of odds ratios (Table 4.2) found no associations between the isolation of *Campylobacter* peri-harvest and isolation on meat.

4.5 Discussion

The prevalence of *Salmonella* fecal shedding in finishing pigs has been reported to average 7.2% nationally (NAHMS), but there is wide variation within individual barns. Thus, the low prevalence we observed in not unexpected or unusual.

The *Campylobacter* prevalence we observed in fecal samples on farm was within the range of prevalence reported by previous authors. The prevalence of *Campylobacter* recovered from the feces of pigs from other studies ranges between 53% to 100% (Oosterom, et al. 1985, Weijtens, et al. 1993, Nesbakken, et al. 2003, Pezzotti, et al. 2003, Oporto, et al. 2007, Thakur, et al. 2005).

There are few reports of collecting pre-fecal material from pigs post-mortem at slaughter. A study conducted in 1985 in England from samples taken post-mortem found a prevalence of 66% (Manser, et al. 1985), which is somewhat lower than our findings (94.6%). One potential explanation is in sample handling. In our study, samples were kept on ice and cultured within 4 hours, while they (Manser, et al. 1985) held samples at ambient temperature for up to 48 hours.

To our knowledge, the present study is the first study to sample porcine hides for *Campylobacter*. The high hide prevalence we observed is likely a reflection of the high fecal prevalence, since fecal contamination on hides is common.

The prevalence of *Campylobacter* we observed on carcasses is much greater than previously reported. A Danish study that included 600 pigs from 152 herds, found the prevalence of *Campylobacter* to be 66% on carcasses measured before chilling (Christensen, et al. 1994). Another study found the prevalence of *Campylobacter* to be 9% and 0% on the carcasses of pigs before and after chilling, respectively (Oosterom, et al. 1985). A study in Belgium found a prevalence of 17% on the carcass ($600cm^2$) (Ghafir, et al. 2007). The study conducted by Thakur and Gebreyes (Thakur, et al. 2005) compared isolation rates of *Campylobacter* on pig carcass's between samples collected pre-evisceration (about 25%) and samples collected post-evisceration (about 50%) and reported a significant difference between prevalence. They also reported a significant reduction of proportion isolated of *Campylobacter* post chill. Blast chilling resulted in the most significant reduction when compared to 4°C chilling overnight. One explanation for the greater proportion of positive carcasses in the present study may be a function of the greater surface area sampled. In this study, one entire carcass side was swabbed whereas, in other studies only a predefined section was sampled. Time from stun to final wash at The Ohio State University Meat Science Laboratory was approximately 40 minutes, which would be a greater time period than observed in some packing plants and which may have contributed to variation in prevalence among trials.

The proportion of positive rib samples contaminated with *Campylobacter* was greater in the present study when compared to other studies conducted using raw pork.

Previous reports indicated prevalence ranging from 1.3% to 18.4%, most measured in the pork chop (Ghafir, et al. 2007, Duffy, et al. 2001, Zanetti, et al. 1996, Korsak, et al. 1998, Ono, et al. 1999, Whyte, et al. 2004, Zhao, et al. 2001, Hong, et al. 2007a, Hong, et al. 2007b, Fricker, et al. 1989). Several studies have been unable to isolate *Campylobacter* from pork (Oosterom, et al. 1985, Ono, et al. 1999, Bohaychuk, et al. 2006, Madden, et al. 1998). One main difference between the methodologies was that we used one pound of ribs incubated in 500 ml of Bolton broth. This is a much larger volume and different cut of meat (most studies used pork chops) than other studies have utilized. The procedures used in the present study may have increased the likelihood of recovering *Campylobacter*. A greater initial material volume, be combined with the location of ribs within the thoracic cavity may result in a greater likelihood of contamination from intestinal contents during evisceration. Ribs were chosen over pork chops for this study due to the perceived greater likelihood of contamination during evisceration and because they are the second most consumed fresh pork product in the US after pork chops (Davis *C.*G., et al. 2005).

The average *Campylobacter* concentrations reported in the present study were higher than reports in the literature for some of sample types/locations. For carcass contamination concentrations, two reports observed between 100 and 1,000 times lower concentration than reported in the present study (Oosterom, et al. 1985). In two studies utilizing pork meat, the concentrations reported were 10 times less than the concentration observed in this study (Wong, et al. 2007). The disparity among reports may be due to the larger surface area sample of carcass swabbed and greater sample weight of meat incubated, resulting in an increased sensitivity in detecting *Campylobacter*. Since the

MPN is calculated by the number of positive plates, an increase in the number of positive plates would result in an increase in the calculated concentration. To the best of our knowledge, this is the only study quantifying *Campylobacter* in feces and on the hide of pigs. The authors of the present study recently completed a similar study in cattle and observed a two-fold increase in the fecal concentration *Campylobacter* of swine manure when compared with fecal samples from cattle. The authors of the present study also observed a one-fold increase in *Campylobacter* concentration in postmortem samples compared with those obtained on the farm when evaluated in cattle at slaughter (Abley, et al.).

Only 500 bacterial cells of *Campylobacter jejuni* have been found to cause clinical signs of Campylobacteriosis in humans (Black, et al. 1988, Deming, et al. 1987, Robinson, et al. 1979). A low infectious dose shows the importance of reducing the prevalence of *Campylobacter* in retail pork samples. Based on our results, a person would need to consume 28g of contaminated raw meat in order to receive an infectious dose, assuming that *C. coli* have a similar infectious dose. Cross-contamination of *Campylobacter* from the raw meat to other surfaces in the kitchen, is also possible and could cause an infection.

4.6 Conclusions

The overall objective of this study was to see if there were any associations between contamination of the final meat product and the samples collected "upstream." We observed a positive correlation between *Campylobacter* concentrations in the farm or lairage pre-fecal samples and concentration of *Campylobacter* on ribs. This suggests that the ribs were contaminated from the intestinal contents of individual animals during

evisceration. It is interesting to note that although there was a measurable association between the concentration in fecal samples and on ribs, *Campylobacter* present in feces did not increase the odds (based on odds ratio) that *Campylobacter* would be recovered from the final meat product. The presence of *Campylobacter* in feces is likely to be important for the risk of meat contamination, but may have been difficult to measure in this population because almost all pigs were positive. The association found between high concentration in feces and concentration on meat suggests that interventions which target the reduction of concentration or identification of high "shedders" should be investigated. Further research should be conducted to elucidate this idea and to generate effective strategies of reducing final meat contamination.

4.7 Acknowledgements

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Figure 4.1 Association between concentration of *Campylobacter* in meat compared to farm, lairage, hide and carcass samples from swine. (Spearman's rank correlation coefficient)

	Spearman's R	P-value
Fecal sample from farm	0.2	0.065
Pre-fecal sample from lairage	0.2	0.068
Hide swab	-0.008	0.95
Carcass swab	0.06	0.66

Table 4.2 Univariate odds Ratios for the risk of *Campylobacter* positive meat sample with the following exposures (farm positive, lairage positive, hide positive or carcass positive) from swine

Risk for *Campylobacter* positive meat samples			
Exposure	OR	95% CI	P-value
Fecal sample from farm	4.5	0.47-219.0	0.2
Pre-fecal sample from lairage	0.33	0.006-4.4	0.6
Hide swab	0.89	0.31-2.6	1
Carcass swab	NA	NA	NA

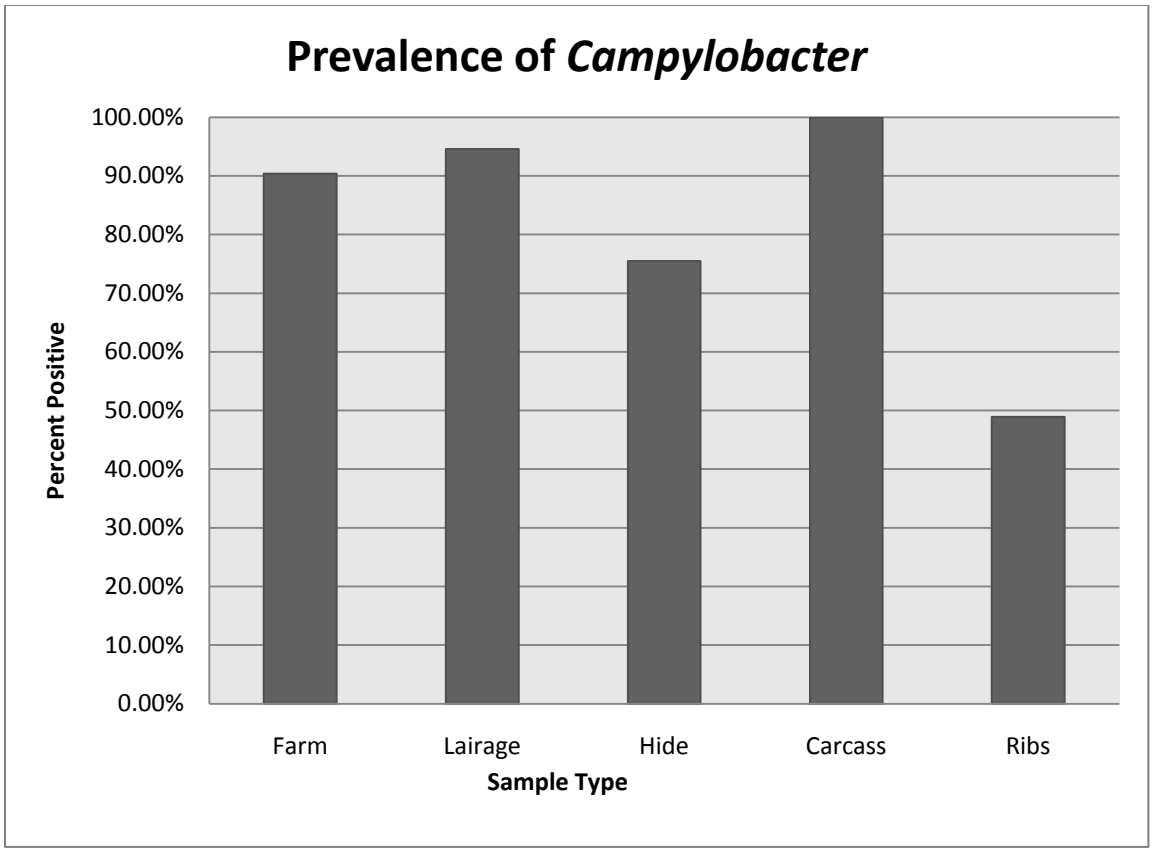

Figure 4.1 The prevalence of *Campylobacter* sp. isolated from the different sample types (farm, lairgae, hide, carcass and meat) in swine N=100

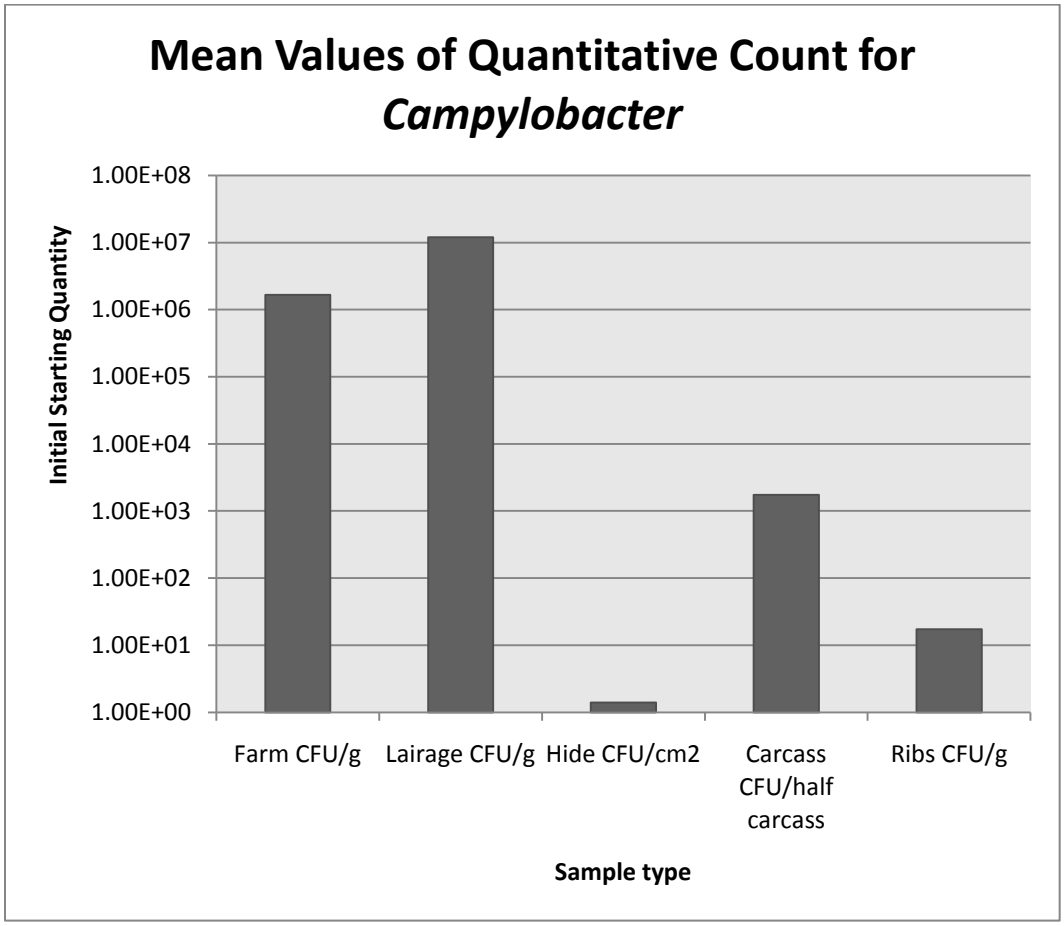

Figure 14.2 The mean value of the quantitative count of *Campylobacter* sp. from all samples (farm, lairge, hide, carcass, and meat) using direct dilution for farm and lairage and MPN for hide, carcass and meat from swine

Chapter 5 Evaluating how quantitative PCR compares to the most probable number method or direct dilution for concentrations of *Campylobacter* in cattle and swine

5.1 Introduction

Campylobacter is a major foodborne pathogen in the United States (Mead, et al. 1999). Swine and cattle often shed this potential pathogen sub-clinically in feces, and fecal contamination of meat during processing can present a food safety risk. Qualitative measures of *Campylobacter* contamination, primarily its presence or absence based on culture, may be used by regulatory agencies to access food safety risk. However, this can be problematic because it does not consider the quantity of bacteria contaminating the product, which is important for the risk of human infection as it relates to infectious dose. Quantitative measurements are particularly important for *Campylobacter jejuni* since the infectious dose as demonstrated by Robinson (Robinson. 1981) was 500 cells, coupled with the observation that *Campylobacter* does not multiply on food at room temperature (Butzler, et al. 1991). Quantitative measures of contamination could be utilized to evaluate interventions and to collect data for public health risk assessments. Quantitative studies are rarely reported in the literature, most likely because of the substantial labor and media requirements of traditional culture based methods for determining pathogen concentration. The objective of this study was to compare the concentration of

Campylobacter using Q-PCR to direct dilution or MPN from 100 swine (5 sample types) and 98 cattle (5 sample types)

5.2 Materials and Methods

5.2.1 Animals Steers

A convenience sample of 98 steers was sampled for this project. All of the steers were reared in the same barn at a single farm owned by The Ohio State University (OSU). The steers were slaughtered at two different facilities, one, which was privately, owned (plant A) and the second owned and operated by OSU (plant B) (Table 5.1).

5.2.2 Animals Swine

A convenience sample of 100 pigs raised together at a single farm owned by OSU were sampled for this study. All of these pigs were harvested at a facility operated by OSU (Table 5.1).

5.2.3 Samples Steers

Within 48 hours of transport to the slaughter facility a fresh fecal sample (~20 g) was obtained from individually identified steers. A fecal sample (~20 g) was also obtained from the individually identified steers immediately post mortem from the rectum. The area of the hide that was the most visually contaminated with feces and dirt was also swabbed immediately post mortem in three different areas of ~910 cm^2 each with three swabs (hydrated-sponge, 3M, Saint Paul, MN). The entire hot carcass was swabbed post-washing and pre-chilling using 5 swabs for each half of the carcass. The following week after the slaughter of each group of steers, an individual ground meat sample from each steer was obtained (~1 pound). All of the swabs were sterile and pre-moistened in 10ml of buffered peptone water.

5.2.4 Samples Swine

Within 48 hours of transport to the slaughter facility, a fresh fecal sample (~20 g) was obtained from individually identified pigs. A fecal sample (~20 g) was also obtained from the individually identified pigs immediately post mortem from the rectum. The visually dirtiest area of the hide was also swabbed while the pigs were in lairage (in three different areas of ~625 cm^2 each with one swab). The entire hot carcass was swabbed post-washing and pre-chilling using three swabs for each half of the carcass. The following week after slaughter ~3 lbs of ribs was obtained from each pig. All of the swabs were sterile and pre-moistened in 10 ml of buffered peptone water.

5.2.5 *Campylobacter* direct dilution and MPN

The fecal samples from farm and fecal samples from lairage were processed by weighing 1g of feces and added to 9ml of BPW. Three dilutions were made taking 1ml from the initial 10^{-1} dilution and adding to 9ml of BPW, etc to a maximum dilution of 10^{-4}. One-hundred microliters from each dilution was plated in duplicate on Campy-Cefex (9) plates and incubated under microaerophilic conditions for 48h at 42° *C.* *Campylobacter* suspect colonies were then counted. The hide swab samples were processed by mixing the sponge with 30ml of Bolton broth (EMD). Ten milliliters of the mixture was added to three tubes and one ml was taken from the 10^{-1} dilution and added to 9ml of Bolton broth to make the next dilution and so on for the 3 tube x 4 dilution MPN. The tubes were incubated under microaerophilic conditions for 48h at 42° *C.* One-hundred microliters from each dilution was plated onto Campy-Cefex plates and incubated under microaerophilic conditions for 48h at 42° *C.* The plates were read yes/no for *Campylobacter* suspect colonies. The 5 sponges (cattle) or 3 sponges (swine) from

the carcass swab were pooled and mixed with 90ml of Bolton broth. A 30ml aliquot of the pooled carcass swab sample was added to each of three tubes and 3ml from the 10^{-1} dilution was added to 27ml of Bolton broth to make a 3 tube x 4 dilution MPN. The samples then followed the protocol for the hide swabs from this point on. Ten grams of ground meat (cattle) or one pound (454 g) of pork ribs was mixed with 90ml (cattle) or 500ml (swine) of Bolton broth and 10ml of this mixture was added to 90ml of Bolton broth to make the 3 tube 4 dilution MPN. The ribs were incubated for 48h at 42° C before the dilutions were made. The samples then followed the protocol for the hide swab and carcass swabs from this point on. Suspect *Campylobacter* colonies from all positive steers from each sample type were saved at -80° C for further processing.

5.2.6 DNA extraction

For Q-PCR, a fecal scoop (stool tube, Sarstedt, Nümbrecht, Germany) ~200mg was used to obtain a fecal sample pre and post spiking. For the meat samples, a 1g sample was taken and placed in 40mL of (BPW). For the carcass swab the piece used for PCR was placed in 80mL of BPW. DNA was extracted from each sample using different methods. DNA from the fecal scoops were extracted using the Qiagen (Valencia, CA) QIAamp DNA Stool Mini Kit according to manufacturer's instructions. After using a two step centrifuge method, 300 rpm to pellet large meat pieces then the supernatant was poured off to be centrifuged for 3,000 rpm to pellet the bacterial cells. The DNA was then extracted from the pellet using the DNeasy Tissue Kit from Qiagen. Forty milileeters from the carcass rinse was centrifuged at 3,000rpm to pellet the bacterial cells then the DNA was extracted from the pellet using the DNeasy Tissue Kit as well.

5.2.7 Q-PCR conditions

Real Time Quantitative PCR was performed on the Mx3005P (Agilent Technologies, Santa Clara, CA). The reactions were at a 25µl final volume. The reaction contained 12.5µl of QuaniTect SYBR Green PCR master mix (Qiagen, Valencia, CA), 1.25µl of extracted DNA (~100ng) and 0.5µM of each primer (Table 5.2). The cycling parameters were not changed ((Hong, et al. 2007)). The standard curve was created by extracting DNA from *Campylobacter jejuni* subsp. *jejuni* ATCC 33560 and *Campylobacter coli* ATCC 49941 the DNA concentration was estimated using a ND-1000 NanoDrop UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE) A stock solution of 1 x 10^7 copies of *Campylobacter* per microliter was prepared for each species. The dilution range of 10^5 to 10^0 was prepared fresh for each PCR using an aliquot of the stock solution. To verify there were no PCR inhibitors five samples from each sample type that were negative initially for QPCR were selected and one microliter of the dilution series of *Campylobacter coli* or *Campylobacter jejuni* from 10^4 to 10^0 prepared for the standard curve was added to each sample and plotted against the standard curve for a spiking experiment.

5.3 Results

5.3.1 MPN

The highest prevalence of *Campylobacter* spp. in steers was from the hide (97%), whereas the lowest prevalence was from the ground beef (12%) (Figure 5.1 and Table 5.3). The highest concentration of *Campylobacter* spp. in steers were up to 9,839,948 cfu/g from the lairage samples and the lowest concentration was from the carcass (142.43 cfu/half carcass) (Figure 5.2 and Table 5.3)

In swine the highest prevalence of *Campylobacter* spp. was from the carcass as all animals sampled were positive, the lowest prevalence was from the ribs at 48.9% (Figure 5.1 and Table 5.4). The highest concentration of *Campylobacter* spp. in swine were from the lairage samples at 27,172,036 cfu/g and the lowest concentrations were also from the carcass at 3,661.1 cfu/half carcass (Figure 5.2 and Table 5.4)

5.3.2 QPCR

The highest prevalence of *Campylobacter jejuni* in steers was from the lairage (8%), whereas the lowest prevalence was from the carcass and ground beef (0%) (Figure 5.1 and Table 5.3). The highest concentration of *Campylobacter jejuni* in steers were up to 99.37 x 10^8 copies/g from the lairage samples and the lowest concentration, from samples that detected *C. jejuni,* was from the hide (0.612 copies/ cm^2) (Figure 5.2 and Table 5.3).

The highest prevalence of *Campylobacter coli* in steers was from the ground beef (18%), whereas the lowest prevalence was from the farm (6%) (Figure 5.1 and Table 5.3). The highest concentrations of *Campylobacter coli* in steers were up to 20,740 copies/g from the farm fecal samples and the lowest concentration was from the carcass (2,2136 copies/half carcass) (Figure 5.2 and Table 5.3).

In swine the highest prevalence of *Campylobacter coli* was from the hide (68%) the lowest prevalence was from the ribs at 0% (Figure 5.1 and Table 5.4). The highest concentration of *Campylobacter coli* in swine were from the lairage samples at 290,800 copies/g and the lowest concentration, from samples that detected *C. coli,* were from the carcass at 2,000 cfu/half carcass (Figure 5.2 and Table 5.4)

5.3.3 Spiking experiment

The results indicate that there were not significant PCR inhibitors present in the extracted composit DNA (Figures 5.3 and 5.4 and Tables 5.5 and 5.6). The Spearman's rank correlation coeffient was 0.9437 for *C. jejuni*, 0.6946 for *C. coli* (steers) and 0.8286 for *C. coli* in pigs all were at a P value of less than 0.00001 indicating a strong positive correlation between the spiked concentration and the actual concentration determined by the standard curve.

5.3.4 Comparison of MPN vs. QPCR

The XY scatter plots and correlation coefficients were all less than or equal to 0.0067 for all the different sample types from both swine and cattle indicate that there was no relationship between the two methods (Figures 5.5 to 5.13).

5.4 Discussion

The prevalence and concentration of *Campylobacter* in pigs appeared higher than that observed in steers. It does not appear that *C. coli* is host specific based on a couple of studies in poultry and cattle (Warner, et al. 1984, Ziprin, et al. 2002). The difference could be that *C. coli* may be shed in higher numbers by pigs than is *C. jejuni*. Other studies have also observed a higher prevalence in swine compared to cattle (Bae, et al. 2005, Stanley, et al. 1998, Rosef, et al. 1983, Hoar, et al. 2001, Cabrita, et al. 1992, Bolton, et al. 1982, Hakkinen, et al. 2007, Giacoboni, et al. 1993, Pezzotti, et al. 2003, Bailey, et al. 2003, Oporto, et al. 2007, Inglis, et al. 2003, Oosterom, et al. 1985, Weijtens, et al. 1993, Nesbakken, et al. 2003, Manser, et al. 1985, Thakur, et al. 2005). Only one other report of C*ampylobacter* quantification in cattle samples is available, but they sampled contents of the small intestine which may not be comparable to our results (Stanley et al., 1998).

There was no observed association when QPCR results were compared to either direct dilution or MPN in cattle or pigs. The discrepancy between the two different quantification methods is difficult to explain. For the fecal samples the difference between direct dilution and QPCR could be that the *Campylobacter* are not distributed evenly throughout the feces therefore the difference in starting volume could make a difference in the recovery of *Campylobacter* when one gram was used for the direct dilution compared to 200mg for the QPCR.

The main difference between the MPN and QPCR was the enrichment step incorporated into the MPN. This enrichment step may have helped in recovering a very small number of viable cells and the QPCR may not have identified the cells due to a smaller starting sample for the PCR. Another possibility is that all of the colonies counted or plates counted as positive for *Campylobacter* may not have actually been *Campylobacter*. For direct dilution and MPN only one colony was saved for further verification of *Campylobacter* due to the large number of samples and labor constraints, therefore the quantitative culture approaches may have artificially inflated the true number of positive colonies or plates.

To address the possibility of PCR inhibitors present in the sample a spiking experiment was conducted and we found that there was not significant inhibition occurring. PCR inhibitors present in feces include bile salts and complex polysaccharides (Lantz, et al. 1997, Monteiro, et al. 1997). PCR inhibitors can react during the PCR reaction in various ways by interfering with the taq polymerase or binding to the DNA or magnesium, the end result is a failed PCR reaction, even if the gene of interest is present or in the case of quantification a lower copy number than what

was really present (Bessetti. 2007). Another study also observed no inhibition by adding a control to the DNA extraction step and they also used the same DNA extraction as was used in this study by (Inglis, et al. 2004, Inglis, et al. 2004).

This study indicates that quantitative culture (direct dilution and MPN) would be the most appropriate means of quantifying *Campylobacter* in cattle and swine samples. This is based on the observation that there was no agreement between the two methods and each of the positive samples for MPN or direct dilution were PCR confirmed thus it appears that the QPCR is producing false negatives suggesting inadequate sensitivity.

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Table 5.10 Slaughter dates and number of animals slaughtered for cattle and swine

Animal	Slaughter Plant	Date	Number
Steer	A	April 3, 2006	10
		April 10, 2006	10
		April 17, 2006	10
		April 24, 2006	10
		May 1, 2006	10
		May 8, 2006	10
	B	April 4, 2006	4
		April 11, 2006	10
		April 18, 2006	8
		April 25, 2006	4
		May 2, 2006	8
		May 9, 2006	4
Pig	B	Oct. 12, 2006	10
		Nov. 2, 2006	10
		Feb. 1, 2007	16
		Feb. 8, 2007	16
		Feb. 15, 2007	16
		Feb. 22, 2007	16
		March 1, 2007	16

Table 5.2 Primers used for the Quantitative Real Time PCR

Primer	Sequence (5'-3')	Bacteria Species	Target gene	Reference
CeuE-F CeuE - R	GATAAAGTTGCAGGAGTTCCAGC TA AACTCCACCTATACTAGGCTTGT CT	*Campylobacter coli*	*ceuE*	(Hong et al., 2007)
HipO-F HipO-R	CTGCTTCTTTACTTGTTGTGGCTT T GCTCCTATGCTTACAACTGCTGA AT	*Campylobacter jejuni*	*hipO*	(Hong et al., 2007)

Table 5.3 The prevalence and range (positive for *Campylobacter*) of *Campylobacter coli* and *Campylobacter jejuni* in steers from all samples (farm, lairage, hide, carcass and meat) for MPN and QPCR

	Campylobacter sp.		***Campylobacter jejuni***		***Campylobacter coli***	
Sample Type	**Prevalence MPN**	**Range MPN**	**Prevalence QPCR**	**Range QPCR**	**Prevalence QPCR**	**Range QPCR**
Farm	78%	500 - 524,416 cfu/g	12%	3.32 – 413.1 copies/g	6%	1,160 – 20,740 copies/g
Lairage	83%	500 - 9,839,948 cfu/g	8%	0.9578 – 9.37 x 10^8 copies/g	8%	841.8 – 1,280 copies/g
Hide	97%	0.0013 – 4.023 cfu/cm^2	4%	0.00107 – 0.612 copies/ cm^2	11%	5.71 x 10^{-5} – 25.8 copies/ cm^2
Carcass	55%	1 – 142.43 cfu/half carcass	0%	NA	16%	253 – 2,2136 copies/half carcass
Ground beef	12%	3.6 – 25.58 cfu/g	0%	NA	18%	2.538 – 1659.8 copies/g

Table 5.4 The prevalence and range of *Campylobacter coli* in swine for all samples (farm, lairage, hide, carcass and meat) for MPN and QPCR

Sample Type	**Prevalence MPN**	**Prevalence QPCR**	**Range MPN**	**Range QPCR**
Farm	90.4%	48%	1,000 – 27,172,036 cfu/g	2.607 – 167,700 copies/g
Lairage	94.6%	58%	500 – 174,464,896 cfu/g	3.183 – 290,800 copies/g
Hide	75.5%	68%	0.00162 – 6.386 cfu/cm^2	3.02 x 10^{-7} – 2.78 copies/cm^2
Carcass	100%	38%	1.19 – 3,661.1 cfu/half carcass	18.58 – 2,040 copies/half carcass
Ribs	48.9%	0%	15.23 – 54,422.3 cfu/g	NA

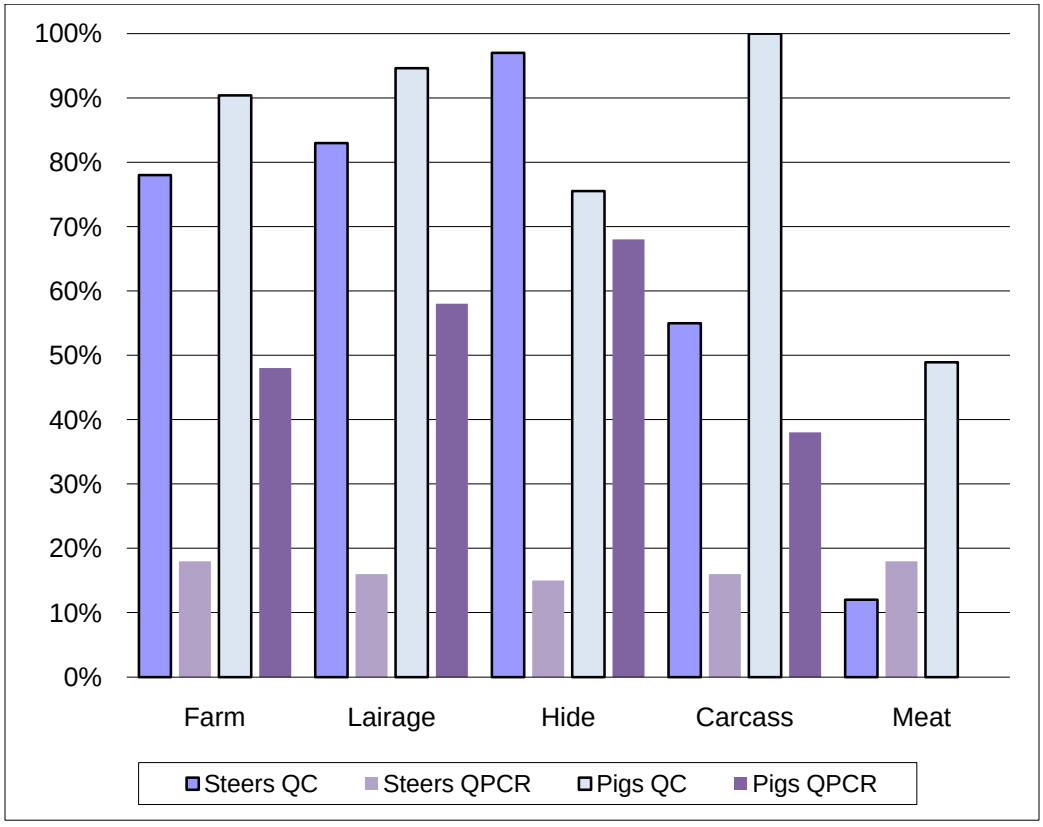

Figure15.1 Proportion of each sample type (farm, lairage, hide, carcass and meat) that was positive for *Campylobacter* sp.

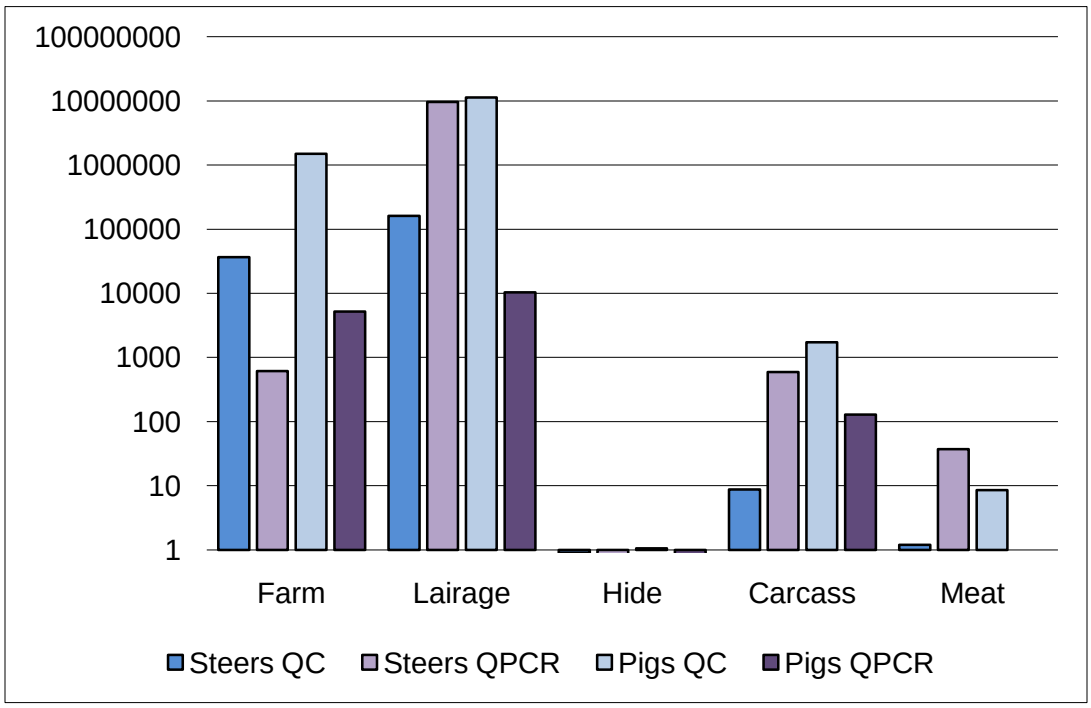

Figure 5.2 The mean quantitative count for *Campylobacter* based on direct diultion and MPN for cattle and swine for each sample type (farm, lairage, hide, carcass and meat)

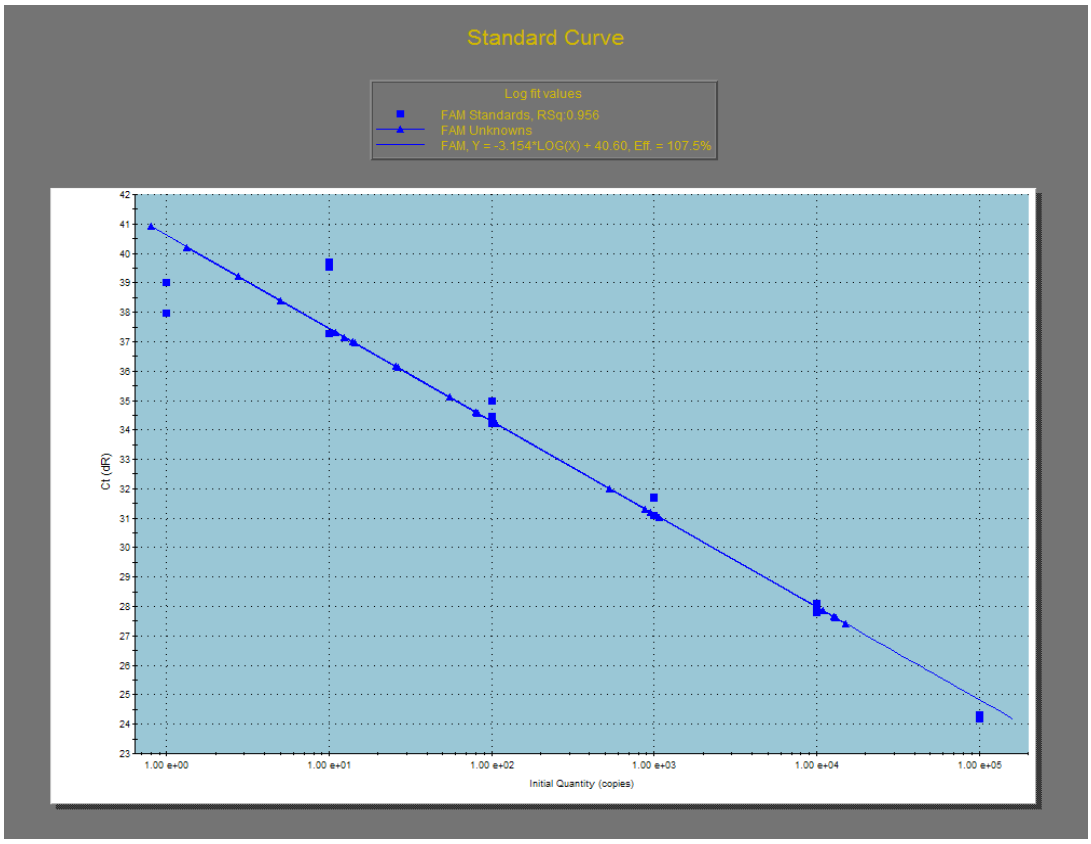

Figure 5.3 Standard Curve for *Campylobacter jejuni* with a selected number of samples to check for PCR inhibitors with in the samples

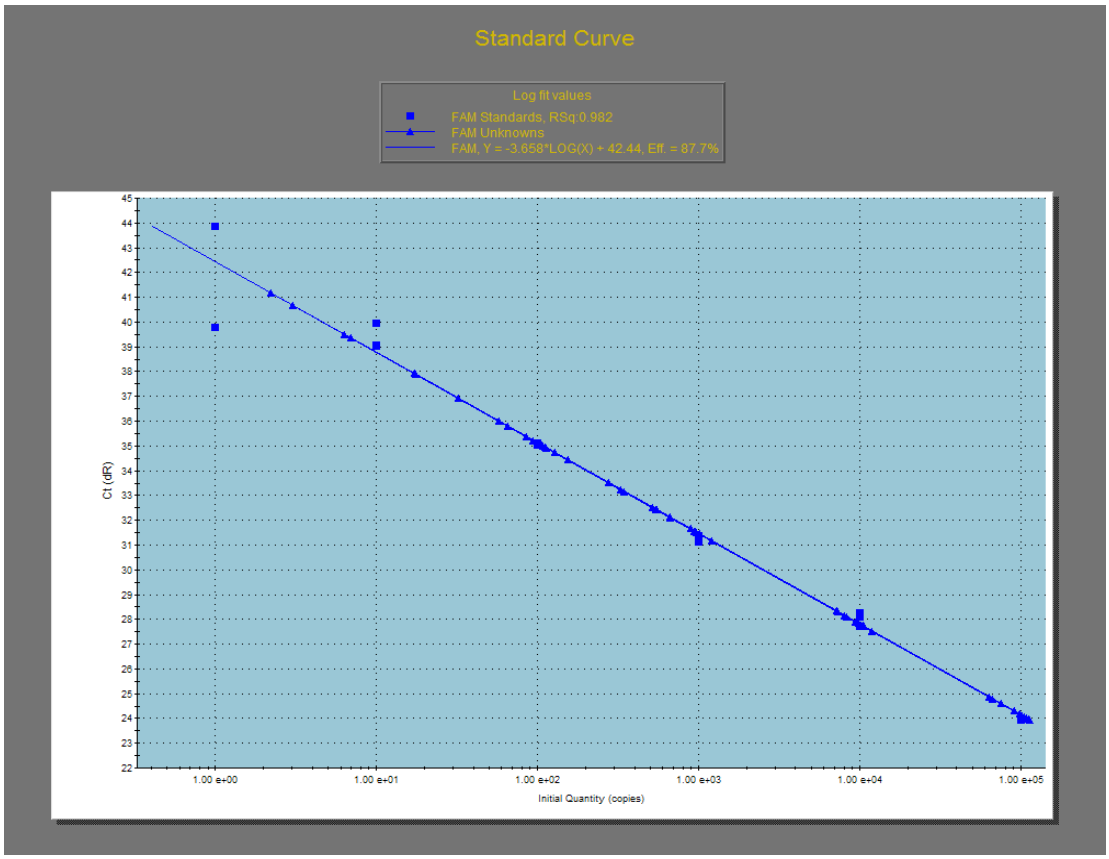

Figure 5.4 Standard curve for *Campylobacter coli* with a selected number of samples to check for PCR inhibitors with in the samples

Table 5.5 *C. jejuni* spiking experiment to address PCR inhibitor presence or absence in steer samples (Spearman's R= 0.9437 $p < 0.00001$)

Sample	Spiked concentration	Actual quantity (copies)
F 17	0.00E+00	7.97E-01
L 17	0.00E+00	1.39E+01
H 17	0.00E+00	0.00E+00
C17	0.00E+00	0.00E+00
M 17	0.00E+00	0.00E+00
H 17	1.00E+00	2.74E+00
L 17	1.00E+00	1.24E+01
C17	1.00E+00	0.00E+00
F 17	1.00E+00	2.57E+01
M 17	1.00E+01	1.10E+01
L 17	1.00E+01	2.64E+01
C17	1.00E+01	4.99E+00
F 17	1.00E+01	1.43E+01
H 17	1.00E+01	1.33E+00
M 17	1.00E+02	1.06E+02
L 17	1.00E+02	7.96E+01
C17	1.00E+02	5.51E+01
F 17	1.00E+02	8.22E+01
H 17	1.00E+02	8.15E+01
L 17	1.00E+03	1.08E+03
C17	1.00E+03	8.85E+02
F 17	1.00E+03	5.34E+02
H 17	1.00E+03	9.55E+02
M 17	1.00E+03	1.04E+03
C17	1.00E+04	1.51E+04
F 17	1.00E+04	1.27E+04
H 17	1.00E+04	1.31E+04
M 17	1.00E+04	1.30E+04
L 17	1.00E+04	1.10E+04

Table15.6 *C.coli* spiking experiment to address PCR inhibitor presence or absence in steer and pig samples (steer Spearman's R= 0.6946 $p < 0.00001$, pig Spearman's R= 0.8286 $p < 0.00001$)

Species	Sample	Spiked concentration	Actual quantity (copies)
Pig	L 16	0.00E+00	3.44E+02
Pig	F16	0.00E+00	3.29E+02
Pig	C16	0.00E+00	1.29E+02
Pig	H16	0.00E+00	1.11E+02
Pig	M 16	0.00E+00	0.00E+00
Pig	C16	1.00E+00	1.28E+02
Pig	L 16	1.00E+00	5.41E+02
Pig	H16	1.00E+00	1.08E+02
Pig	F16	1.00E+00	3.25E+01
Pig	M 16	1.00E+00	6.36E+00
Pig	F16	1.00E+01	1.21E+03
Pig	H16	1.00E+01	9.72E+02
Pig	L 16	1.00E+01	5.16E+02
Pig	C16	1.00E+01	1.55E+02
Pig	M 16	1.00E+01	9.45E+01
Pig	H16	1.00E+02	1.20E+04
Pig	F16	1.00E+02	1.02E+04
Pig	L 16	1.00E+02	1.21E+03
Pig	M 16	1.00E+02	1.01E+03
Pig	C16	1.00E+02	5.52E+02
Pig	H16	1.00E+03	1.02E+05
Pig	F16	1.00E+03	7.52E+04
Pig	C16	1.00E+03	1.04E+04
Pig	L 16	1.00E+03	9.88E+03
Pig	M 16	1.00E+03	9.42E+03
Pig	L 16	1.00E+04	1.14E+05
Pig	M 16	1.00E+04	1.08E+05
Pig	C16	1.00E+04	9.88E+04
Pig	F16	1.00E+04	2.77E+02
Pig	H16	1.00E+04	1.13E+02
Steer	C 17	0.00E+00	0.00E+00
Steer	F 17	0.00E+00	0.00E+00
Steer	H 17	0.00E+00	0.00E+00
Steer	L 17	0.00E+00	0.00E+00
Steer	M 17	0.00E+00	0.00E+00
Steer	M 17	1.00E+00	1.75E+01
Steer	C 17	1.00E+00	1.72E+01
Steer	L 17	1.00E+00	6.94E+00
Steer	F 17	1.00E+00	3.05E+00
Steer	H 17	1.00E+00	2.20E+00

Continued

Table 5.6 Contiuned

Species	Sample	Spiked concentration	Actual quantity (copies)
Steer	F 17	1.00E+01	9.60E+02
Steer	C 17	1.00E+01	1.07E+02
Steer	M 17	1.00E+01	8.47E+01
Steer	H 17	1.00E+01	6.51E+01
Steer	L 17	1.00E+01	5.75E+01
Steer	F 17	1.00E+02	8.04E+03
Steer	M 17	1.00E+02	9.43E+02
Steer	C 17	1.00E+02	8.90E+02
Steer	H 17	1.00E+02	6.67E+02
Steer	L 17	1.00E+02	6.65E+02
Steer	F 17	1.00E+03	1.05E+05
Steer	M 17	1.00E+03	1.06E+04
Steer	C 17	1.00E+03	8.27E+03
Steer	H 17	1.00E+03	7.27E+03
Steer	L 17	1.00E+03	7.20E+03
Steer	M 17	1.00E+04	1.12E+05
Steer	C 17	1.00E+04	9.14E+04
Steer	L 17	1.00E+04	6.72E+04
Steer	H 17	1.00E+04	6.32E+04
Steer	F 17	1.00E+04	0.00E+00

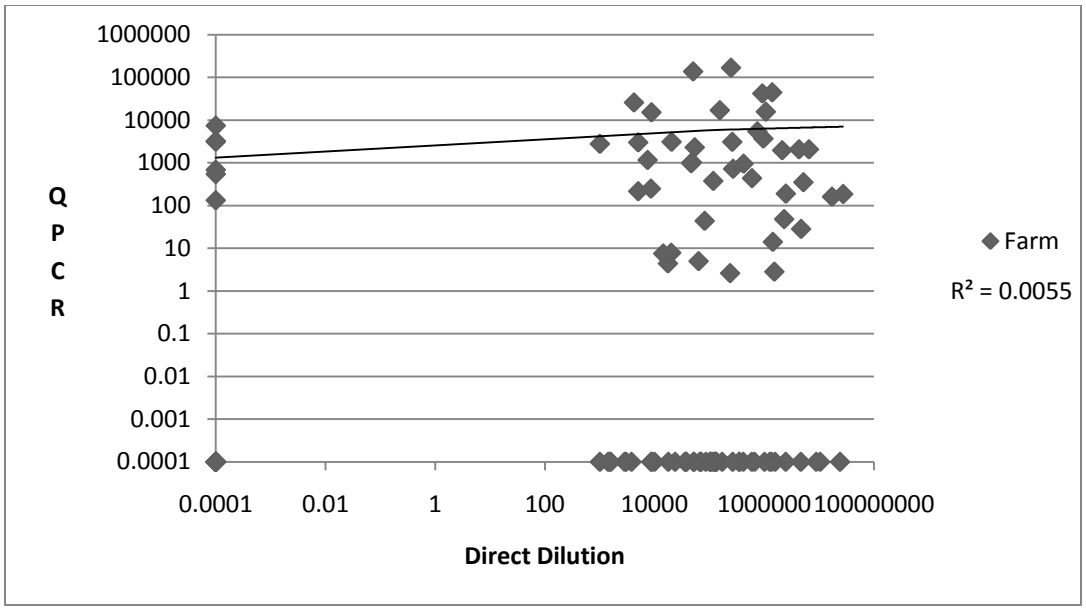

Figure 5.5 Quantitative PCR vs. direct dilution of *Campylobacter coli* in swine on farm

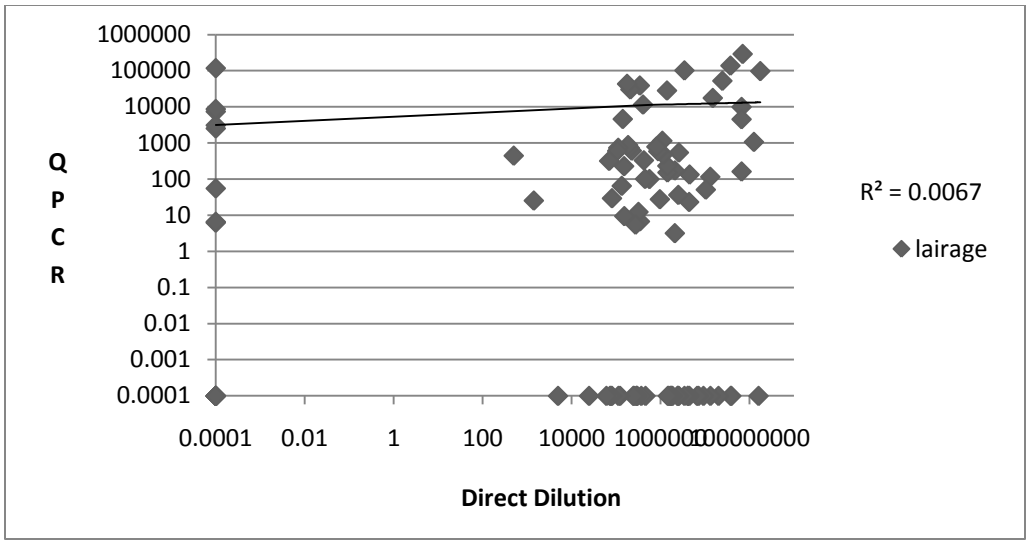

Figure 5.6 Quantitative PCR vs. direct dilution of *Campylobacter coli* in swine from lairage

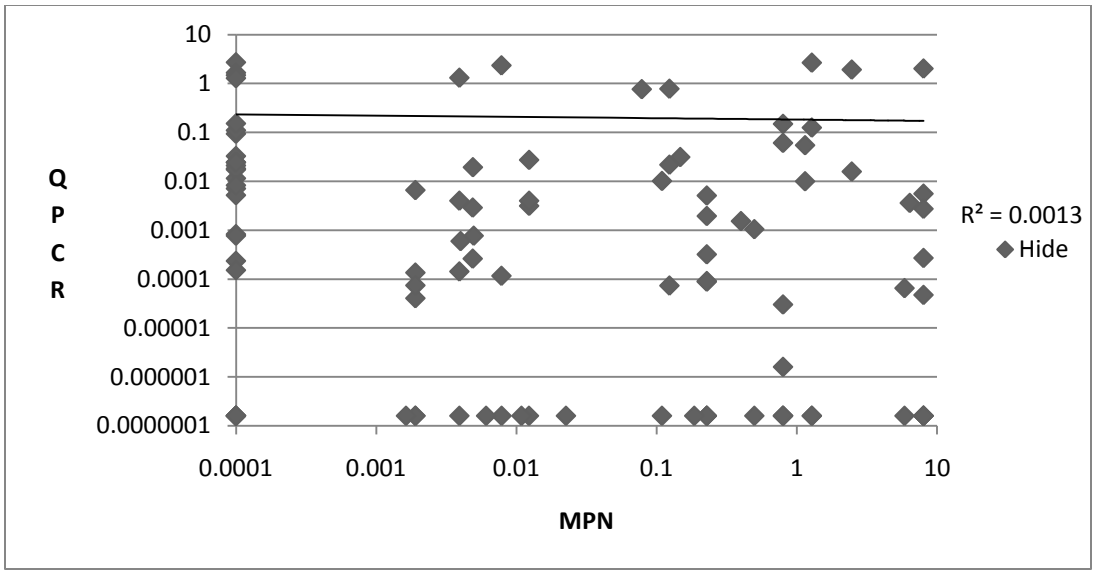

Figure 5.7 Quantitative PCR vs. direct dilution of *Campylobacter coli* in swine from hide samples

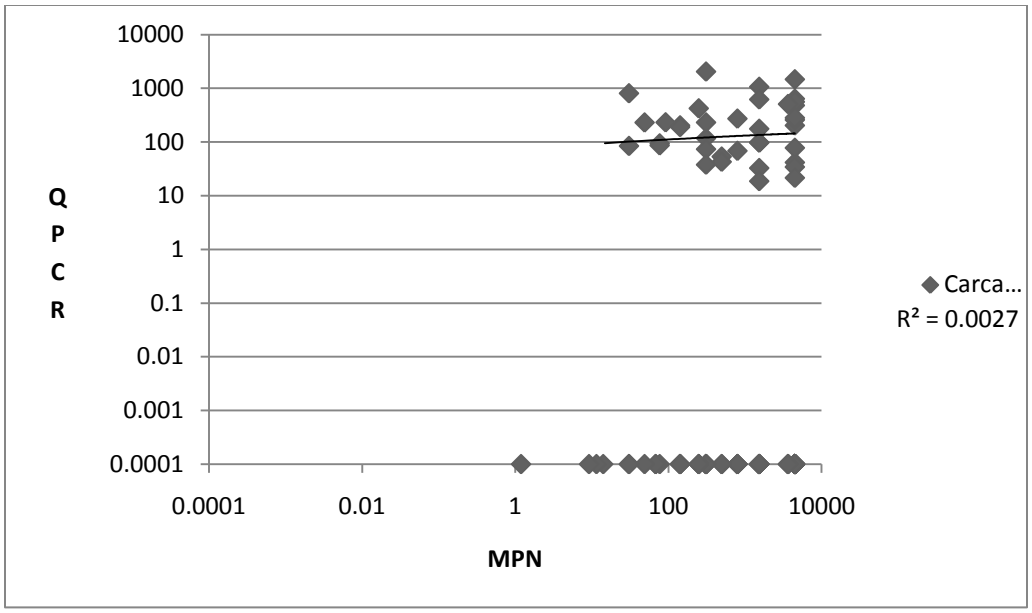

Figure 5.8 Quantitative PCR vs. direct dilution of *Campylobacter coli* in swine from carcass samples

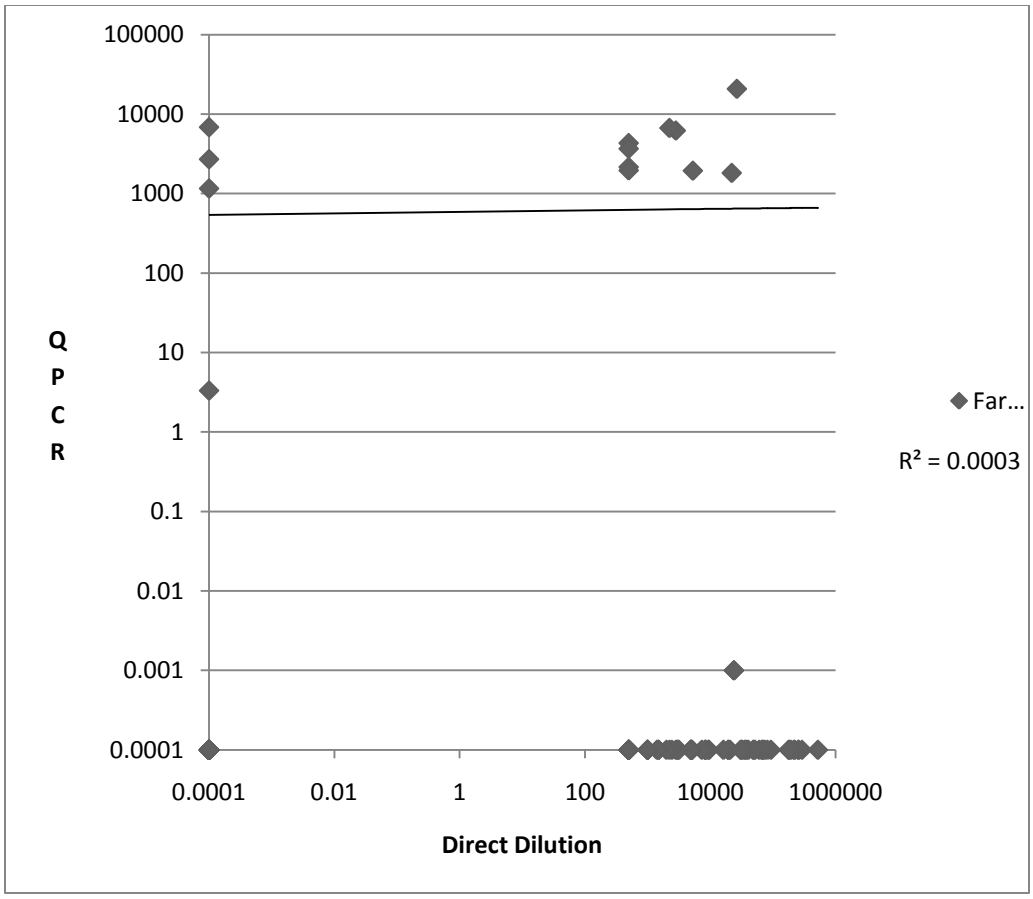

Figure 5.9 Quantitative PCR vs. direct dilution of *Campylobacter coli* and *Campylobacter jejuni* in cattle from farm

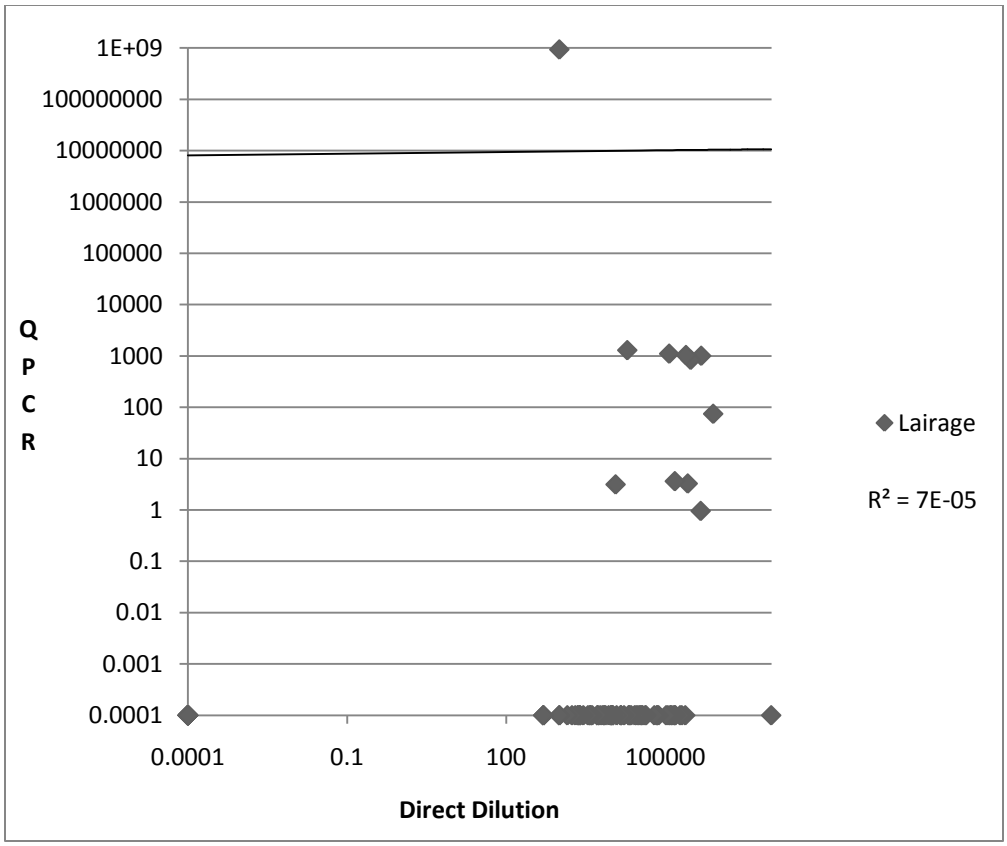

Figure 5.10 Quantitative PCR vs. direct dilution of *Campylobacter coli* and *Campylobacter jejuni* in cattle from lairage

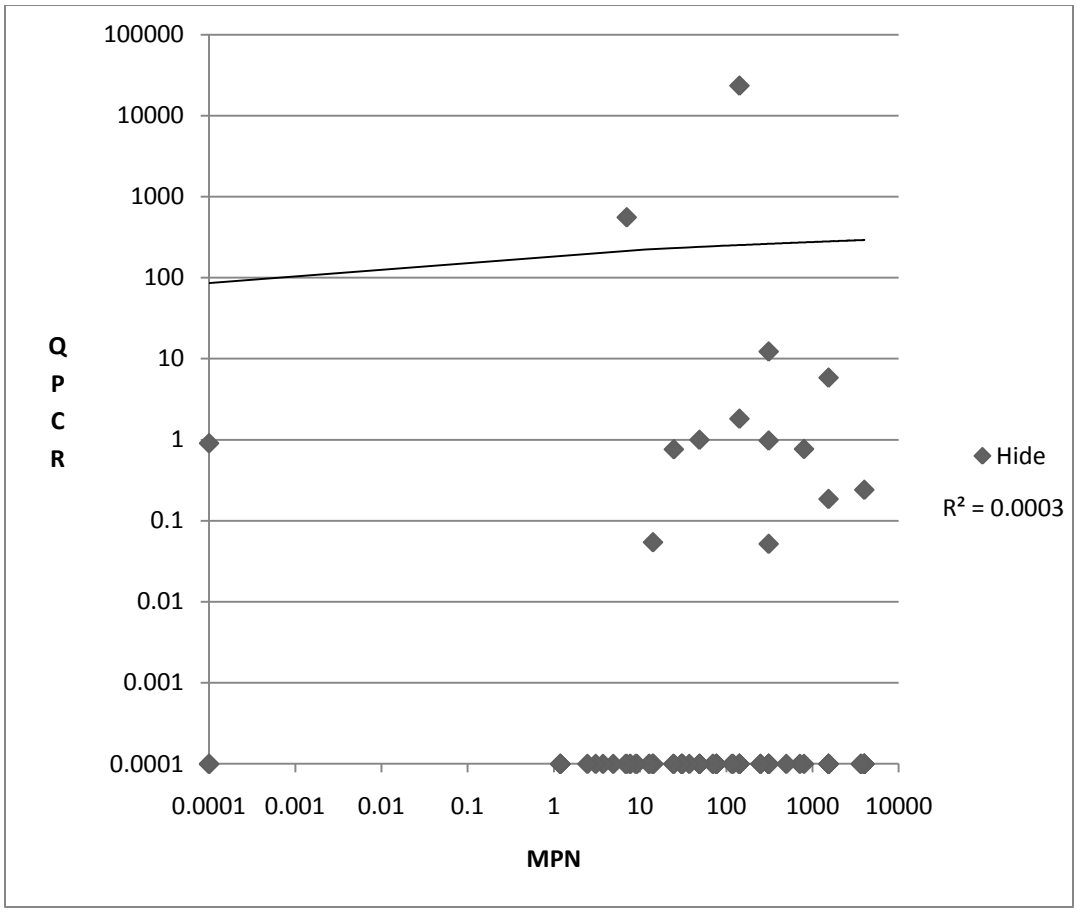

Figure 5.11 Quantitative PCR vs. MPN of *Campylobacter coli* and *Campylobacter jejuni* in cattle from hide

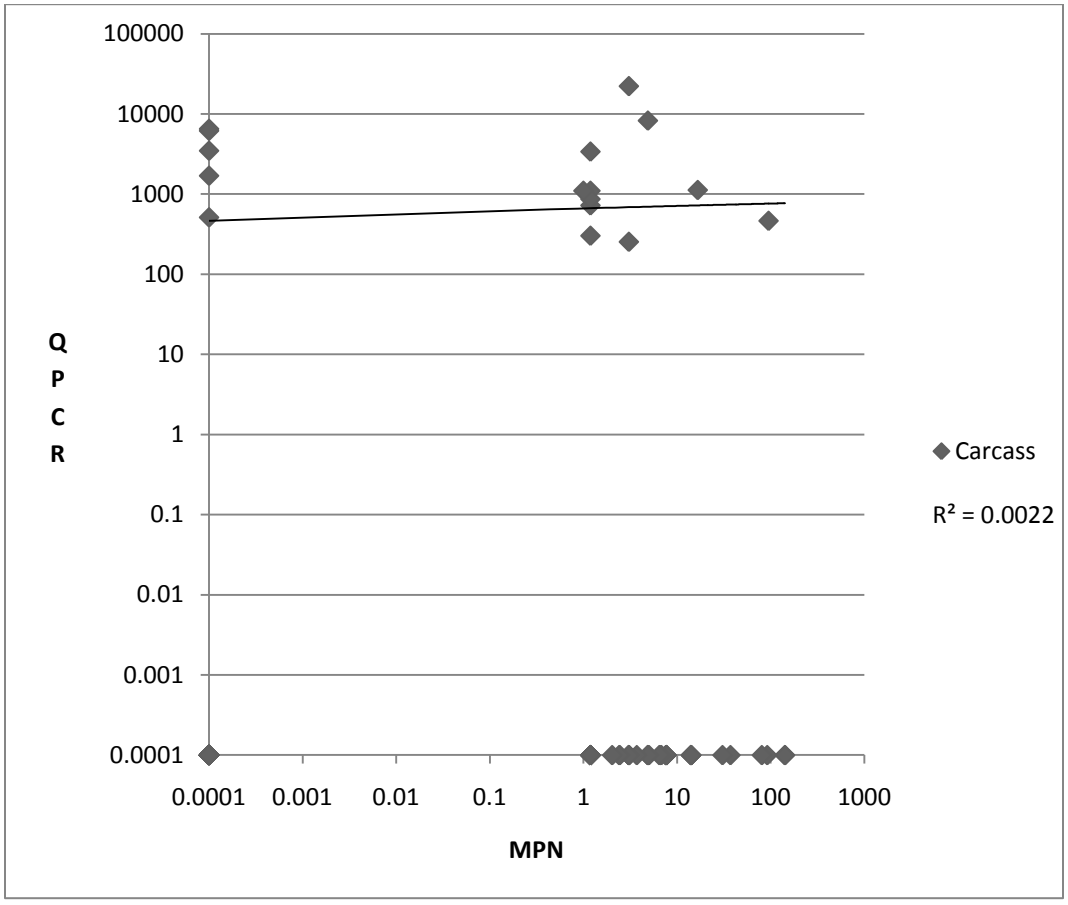

Figure 5.12 Quantitative PCR vs. MPN of *Campylobacter coli* and *Campylobacter jejuni* in cattle from carcass

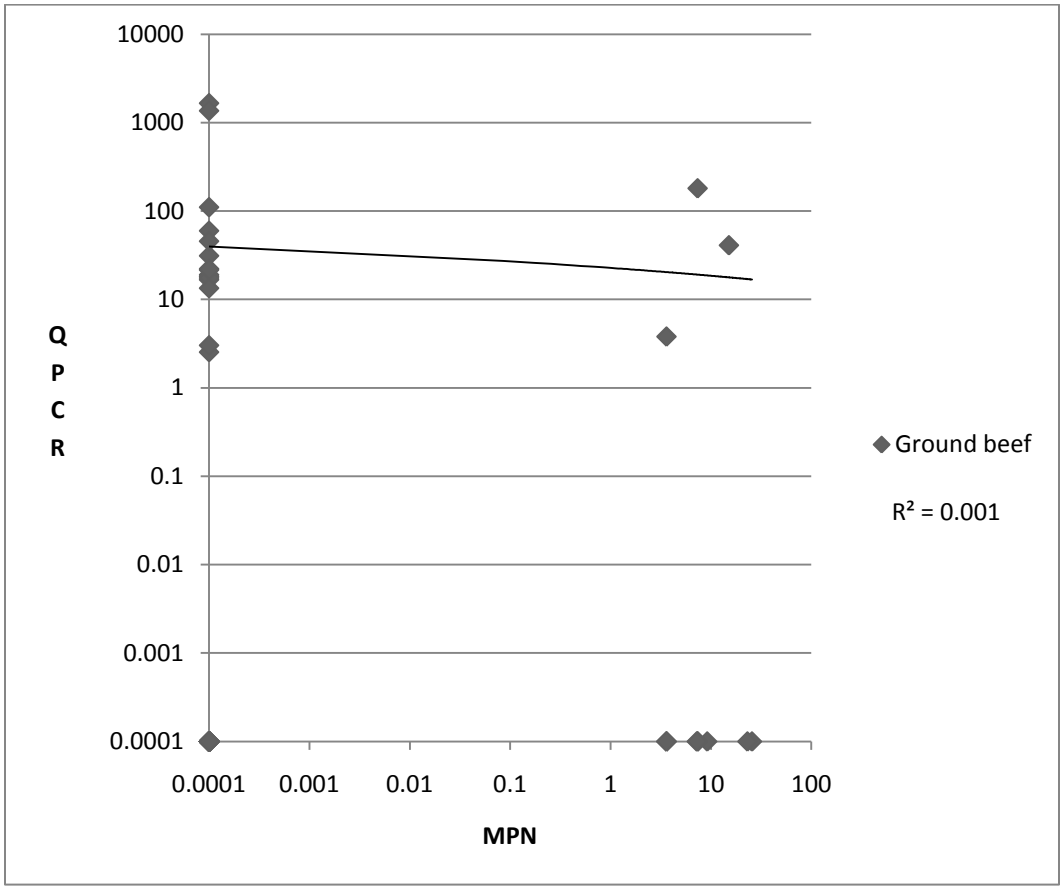

Figure 5.13 Quantitative PCR vs. MPN of *Campylobacter coli* and *Campylobacter jejuni* in cattle from ground beef

Chapter 6 Antimicrobial Susceptibility and Multi-Locus Sequence Typing of *Campylobacter coli* in swine before, during and after the slaughter process[3]

6.1 Abstract

The objective of this study was to determine the clonal relatedness of *Campylobacter coli* from pigs on farm and at processing. We conducted antimicrobial susceptibility and multi-locus sequence typing (MLST). *Campylobacter* isolates from farm, lairage, hide and carcass showed similar phenotypes and belonged to the same clonal groups based on MLST. Five new Sequence Types were identified (ST-4083, ST-4084, ST-4085, ST-4086, ST-4087). This study found a high diversity of *C. coli* within one farm and meat was contaminated with the same STs as isolates collected from previous stages in the harvest process.

6.2 Introduction

Approximately 1.4–2.3 million persons are infected with *Campylobacter* annually in the United States, and most of these illnesses are food-related (Samuel, et al. 2004). Consumption of contaminated meat, milk, and water have been implicated as primary sources of *Campylobacter* infection (Frost. 2001, Deming, et al. 1987, Kapperud, et al. 1992, Hopkins, et al. 1984, Oosterom, et al. 1984, Sacks, et al. 1986, Zhao, et al. 2001,

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Thakur, et al. 2006, Lévesque, et al. 2007, Litrup, et al. 2007, Kwan, et al. 2008). Pigs are often non-clinically infected with *Campylobacter* and contamination of meat during processing remains a food safety risk. Campylobacteriosis in humans may be further complicated by the emergence of antimicrobial resistant strains that may limit therapeutic options. Although *C. jejuni* is considered the most important *Campylobacter* species causing infection in humans, recent studies have highlighted the importance of *C. coli* as a food-borne pathogen that is frequently resistant to antimicrobials (Englen, et al. 2005, Gillespie, et al. 2002, Sails, et al. 2003, Tam, et al. 2003, Bywater, et al. 2004).

Phenotypic and genotypic approaches are routinely implemented to characterize *Campylobacter*. MLST is a highly discriminatory DNA fingerprinting method that has proven useful in organisms with a hypervariable genome including *Campylobacter* (Dingle, et al. 2002). There are no reports of comparative clonal relatedness of *Campylobacter* from the same animal from different stages along the food processing chain. This is especially important in defining the role of pre- harvest (on farm, in lairage) factors for contamination of meat. The objective of this study was to determine the phenotypic and genotypic relatedness of *C. coli* isolates from the same pigs collected from the farm through processing.

6.3 Materials and Methods

6.3.1 Study design

Isolates from this study originated from a previous unpublished study. One hundred pigs were individually identified from one farm and followed within 7 cohorts (10-16/cohort) through the harvest process. Samples collected from each pig included a fecal sample on farm (farm), rectal contents post-eviseration (lairage), a hide swab in

lairage (hide), carcass swabs immediately after wash and pre-chilling (carcass) and a meat sample (ribs). Inclusion criteria for this study were for pigs from which a *C. coli* was isolated from each of these sample types. Eleven pigs (55 isolates) met this criterion.

6.3.2 *Campylobacter* Culture

Fecal and rectal samples were cultured without pre-enrichment. One gram of feces was diluted in 9 ml of BPW and 100 µl were plated in duplicate on Campy-Cefex (Oyarzabal, et al. 2007)(LaGier, et al. 2004) plates. The remaining swab and meat samples were enriched in Bolton broth followed by plating. The dilution for the samples were: hide swab in 30 ml, carcass swabs (5 pooled) in 90 ml and 1 pound (454 g) of rib meat in 500 ml All broth enrichments were incubated under microaerophilic conditions for 48 h at 42*C.* One-hundred µl from each enrichment was plated onto a Campy-Cefex plate and incubated under microaerophilic conditions for 48h at 42° *C.* Biochemical confirmation was done using Catalase (Becton Dickenson) and Oxidase (Becton Dickenson) tests. DNA extraction was done using the DNeasy Tissue kit (Qiagen). PCR was performed on the extracted DNA targeting the *hipO* gene for *Campylobacter jejuni* and the *glyA* gene for *Campylobacter coli* (LaGier, et al. 2004).

6.3.3 Antimicrobial Susceptibility

Antimicrobial susceptibility of isolates was determined by using an approved standard broth microdilution method (Anonymous 2006). The experiment used commercially prepared CAMPY plates (Sensititre, TREK™ Diagnostic Systems In*C.*, Westlake OH) and samples were tested according to the manufacturer's instructions. Breakpoint values were based on Clinical and Laboratory Standards Institute and by the

United States National Antibiotic Monitoring System (NARMS) as shown on Table 6.1. Minimum inhibitory levels were determined using the SensiTouch (TREK™ Diagnostic Systems Inc). The quality control organism *C. jejuni* ATCC 33560 was used to verify the quality of the plates (Anonymous 2006).

6.3.4 Multi-Locus Sequence Typing

MLST was performed on the purified DNA for each of the following seven housekeeping genes as described (Dingle, et a. 2005): *aspA* (aspatase), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxy methyl transferase), *pgm* (phospho glucomutase), *tkt* (transketolase), *uncA* (ATP synthase alpha subunit). Ready-to-Go PCR beads (GE Healthcare Life Sciences, Piscataway, NJ) were used for all PCR amplifications. PCR reaction conditions were initial denaturation at 95° C for 5 min, followed by 30 cycles of the following, 95° C for 1 min, primer annealing at 55° C for 90 sec, and extension at 72° C for 1 min. The PCR products were purified by using a Qiaquick multiwell PCR purification kit (Qiagen). Sequencing reactions were conducted in a volume of 20 µl containing 1 µl purified PCR product, 2 µl primer (10 pmol/µl), 1.5 µl sequencing buffer (Beckman Coulter, Fullerton, CA), 2 µl DTCS Quick Start Master Mix (Beckman Coulter), and 13.5 µl molecular grade water. Thermal cycling conditions for sequencing reactions were set up according to the manufacturer's (Beckman Coulter) instructions. Unincorporated dye terminators were removed by ethanol precipitation, and the sequenced products were separated and detected with a CEQ 8000 Genetic Analysis System (Beckman Coulter). Alleles and sequence types (STs) were assigned by submitting each DNA sequence to the *Campylobacter* MLST database (http://pubmlst.org/*Campylobacter*). A dendrogram was created using Bionumerics

software version 4.0 (Applied Maths, Kortrijik, Belgium). Additionally, the ClustalW software (http://www.ebi.a*C*.uk/Tools/msa/clustalw2/) was used to perform the sequence alignments.

6.4 Results and Discussion

The most common antimicrobial resistance phenotypes for the five sample types were farm, pansusceptible [n=3], and Te [n=2]; lairage, ArCaErTe [n=3]; hide, Te [n=7]; carcass, Te [n=7]; and meat Te [n=2] (Table 6.2). The high frequency of resistance to tetracycline (77%) and erythromycin (28%) were similar results previously reported by our group (Abley et al.).

Campylobacter isolates from meat were phenotypically similar and are within the same MLST clonal complex (Figure 6.2) as isolates collected from farm, lairage, hide and carcass. This indicates that the isolates on the meat most likely originated from contamination from the feces or hide of the pigs.

Five new Sequence Types (STs) were identified (ST-4083, ST-4084, ST-4085, ST-4086, and ST-4087). There were 13 different sequence types identified from the 43 isolates successfully recovered from cryopreservation ST-854 (n=14) and ST-1056 (n=10) were the most common and they included samples from the farm, lairage, hide, carcass and meat, further strengthening the evidence of clonal distribution along the farm to food continuum (Figure 6.1). It is also interesting that some STs exhibited multiple resistance patterns. For instance, ST1056 exhibited patterns including pansusceptible, Te or multi-drug resistance with ArCaNlTe. ST854 predominantly exhibited Pan-susceptible or Te. This is not entirely unexpected, since MLST genotyping focuses of housekeeping genes whereas antimicrobial resistance could be encoded in regions with greater

plasticity including extra chromosomally on plasmids. However, this emphasizes the importance of using a combination of phenotype and genotype to fully characterize *Campylobacter* isolates for epidemiologic classification. A study conducted by Miller et al. (Miller, et al. 2007) typed a total of 488 *C. coli* strains from four different food sources (cattle, chicken, swine and turkeys). Of the four sources, they observed that swine had the most diverse STs, with 82 unique STs identified from their 185 swine samples (Miller, et al. 2007). Our results however, found a higher diversity of isolates originating from only 11 pigs. When comparing our isolate STs to those recovered by Miller *et al*., (Miller, et al. 2007) several STs were identical (ST-854, ST-828, and ST-1107). We previously reported (Thakur, et al. 2005) diversity of *Campylobacter* as high as 65 unique STs recovered from 100 swine isolates. The most common STs we previously reported that were also present in the current study were ST-854, ST-828, and ST-1056. There appears to be a high diversity of STs among *C. coli* isolated from swine.

Our results indicate that clonal *C. coli* isolates can be recovered from feces, hide, carcass, and final fresh meat product of the same animal. This result implies that identification of effective on-farm interventions to reduce *C. coli* infection of pigs are likely to result in reduced contamination of fresh pork products.

6.5 Acknowledgements

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of Oxford (Jolley & Maiden 2010, BMC Bioinformatics, 11:595)

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Table16.1 Minimum inhibitory concentrations used as breakpoints for susceptibility testing1 of *Campylobacter coli* isolates recovered from feces, colon, hide, carcass, and final meat product from 11 finishing swine

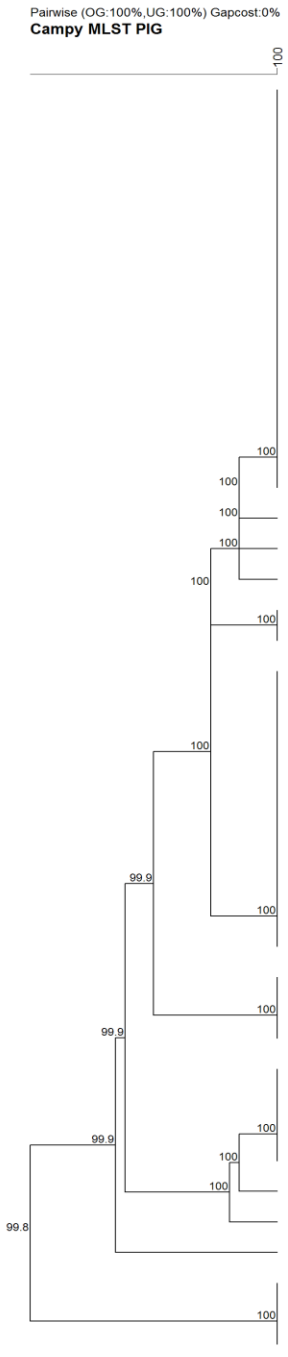
Antimicrobial Class	**Antimicrobial Agent**	**Breakpoints (µg/ml)**		
		Susceptible	**Intermediate**	**Resistant**
Aminoglycosides	Gentamicin	≤ 2	4	≥ 8
Ketolides	Telithromycin	≤ 4	8	≥ 16
Lincosamides	Clindamycin	≤ 2	4	≥ 8
Macrolides	Azithromycin	≤ 2	4	≥ 8
	Erythromycin	≤ 8	16	≥ 32
Phenicols	Florfenicol[2]	≤ 4	N/A	N/A
Quinolones	Ciprofloxacin	≤ 1	2	≥ 4
	Nalidixic acid	≤ 16	32	≥ 64
Tetracyclines	Tetracycline	≤ 4	8	≥ 16

[1]Breakpoints were adopted from CLSI (Clinical and Laboratory Standards Institute), when not available they were adopted from the National Antimicrobial Resistance Monitoring System (NARMS).
[2]For florfenicol, only a susceptible breakpoint (≤ 4 µg/ml) has been established. In this study, isolates with a MIC ≥ 8 µg/ml are categorized as resistant.
Table adopted from USDA, ARS, BEAR, 2007 Veterinary Isolates Final Report (1)

Table 6.2 Antimicrobial Resistance Patterns of the *Campylobacter coli* isolates recovered from feces, colon, hide, carcass, and final meat product from 11 finishing swine

Pig	Farm	Lairage	Hide	Carcass	Meat
2	ArCaErGmNlTe	Te	Te	Te	No growth
3	Te	Pan Susceptible	Te	Te	ArErTe
4	ArCaErFfTe	ArCaErTe	Te	Te	No growth
6	Pan Susceptible	ArCaErTe	Ca	Te	No growth
7	Pan Susceptible	No growth	Pan Susceptible	Pan Susceptible	No growth
10	ErNlTe	No growth	Pan Susceptible	Te	ArGmNlTe
26	No growth	ArCaErNlTe	Te	Te	Pan Susceptible
31	ArCaErTe	ArCaErFfNlTe	Te	Pan Susceptible	ArCaErTe
43	CIPGmNlTe	ArCaErTe	Te	Te	Te
76	Te	CaErTe	Te	No growth	No growth
85	Pan Susceptible	No growth	No growth	No growth	Te

(Ar – Azithromycin, Ca – Clindamycin, CIP – Ciprofloxacin, Er – Erythromycin, Ff – Florfenicol, Gm – Gentamycin, Nl – Naladixic acid, Tt – Telithromycin, Te – Tetracycline)



Key	Pig	Source	Sequence Type	R-Pattern	Date Harvested
C6972	4	Farm	ST-854	ArCaErFfTe	Oct. 12, 2006
C6973	10	Farm	St-854	ErNlTe	Oct. 12, 2006
C6975	43	Farm	ST-854	ClPGmNlTe	Feb. 8, 2007
C6978	6	Lairage	ST-854	ArCaErTe	Oct. 12, 2006
C6982	7	Carcass	ST-854	Pan Susceptable	Oct. 12, 2006
C6985	7	Farm	ST-854	Pan Susceptable	Oct. 12, 2006
C6987	85	Farm	St-854	Pan Susceptable	March 1, 2007
C6989	26	Carcass	ST-854	Te	Feb. 1, 2007
C6990	31	Carcass	ST-854	Pan Susceptable	Feb. 1, 2007
C6994	4	Hide	ST-854	Te	Oct. 12, 2006
C6995	3	Hide	ST-854	Te	Oct. 12, 2006
C7030	6	Farm	ST-854	Pan Susceptable	Oct. 12, 2006
C7032	6	Hide	ST-854	Ca	Oct. 12, 2006
C7033	7	Hide	ST-854	Pan Susceptable	Oct. 12, 2006
C6966	31	Meat	ST-1579	ArCaErTe	Feb. 1, 2007
C6996	2	Hide	ST-1556	Te	Oct. 12, 2006
C7034	10	Hide	ST-828	Pan Susceptable	Oct. 12, 2006
C6970	3	Meat	ST-1107	ArCaErNlTe	Oct. 12, 2006
C7031	31	Farm	ST-1107	ArCaErTe	Feb. 1, 2007
C6964	10	Meat	ST-1056	ArGmNlTe	Oct. 12, 2006
C6965	26	Meat	ST-1056	Pan Susceptable	Feb. 1, 2007
C6967	43	Meat	ST-1056	Te	Feb. 8, 2007
C6976	2	Lairage	St-1056	Te	Oct. 12, 2006
C6981	26	Lairage	ST-1056	ArCaErNlTe	Feb. 1, 2007
C6983	3	Lairage	ST-1056	Pan Susceptable	Oct. 12, 2006
C6991	43	Carcass	ST-1056	Te	Feb. 8, 2007
C7037	43	Hide	ST-1056	Te	Feb. 8, 2007
C7040	4	Carcass	ST-1056	Te	Oct. 12, 2006
C7041	85	Meat	ST-1056	Te	March 1, 2007
C6958	31	Lairage	ST-4083	ArCaErFfNlTe	Feb. 1, 2007
C6959	43	Lairage	ST-4084	ArCaErTe	Feb. 8, 2007
C6960	76	Lairage	ST-4084	CaErTe	Feb. 22, 2007
C6986	3	Farm	ST-4086	Te	Oct. 12, 2006
C6993	76	Hide	ST-4086	Te	Feb. 22, 2007
C7038	2	Carcass	ST-4086	Te	Oct. 12, 2006
C7039	3	Carcass	ST-4086	Te	Oct. 12, 2006
C6971	2	Farm	ST-1436	ArCaErGmNlTe	Oct. 12, 2006
C7035	26	Hide	ST-4087	Te	Feb. 1, 2007
C7036	31	Hide	ST-4088	Te	Feb. 1, 2007
C6984	76	Farm	ST-4085	Te	Feb. 22, 2007
C6988	10	Carcass	ST-4085	Te	Oct. 12, 2006
C6992	6	Carcass	ST-4085	Te	Oct. 12, 2006

Figure 6.1 Dendrogram built by pairwise comparison of the consensus sequences from each of the seven allele sequences for the 47 *Campylobacter coli* isolates recovered from feces, colon, hide, carcass, and final meat product from 11 finishing swine

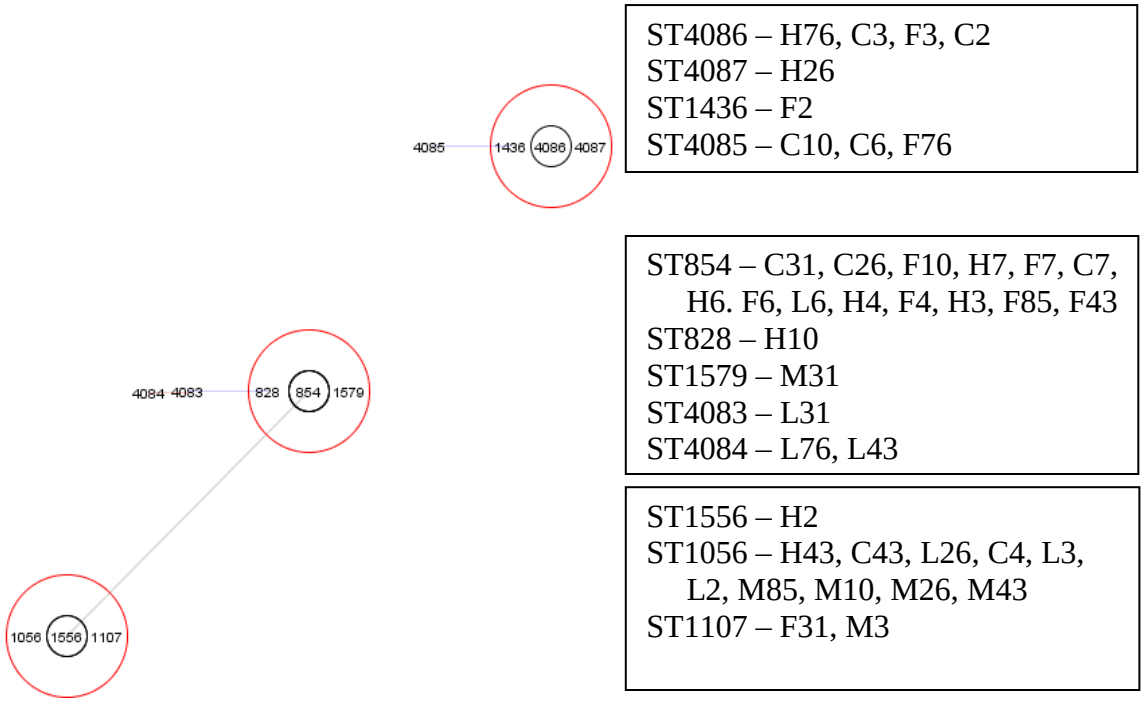


Figure 6.29 Based upon related sequences (BURST) analysis for all the sequence types. The sequence type in the center is the founding sequence type for each of the three clonal complexes. The sequence types in the red circle indicate single locus deviants (SLD) and the lines represent double locus variants (DLV).

Chapter 7 Discussion

7.1 Chapter 2 Comparison of Direct Dilution, Most Probable Number estimation, and Quantitative real-time PCR for enumeration of *Salmonella* and *Campylobacter* in spiked cattle and pig samples

- QPCR indicated there was a strong positive correlation between the concentration of *Campylobacter* pre and post spiking for all the sample types tested.
- *Salmonella* MPN results indicated there was a strong positive correlation between the pre- and post-spiking concentrations for the MPN results from the bovine, and porcine fecal samples.
- *Campylobacter* direct dilution in bovine feces resulted in a strong positive correlation between initial spiking concentrations and post-spiking values.
- *Campylobacter* MPN results indicated there was a strong positive correlation between the pre and post spiked samples for carcass and ground beef samples.
- Q-PCR provided good correlation between pre- and post-spiked samples for *Salmonella*, therefore Q-PCR will be a benefical tool for quantification of *Salmonella* from fecal, carcass and meat samples..
- Our results help to differentiate the relative value of the existing methods for quantification of *Campylobacter,* and emphasize the need for new and improved methods.

- Q-PCR appears to be the best method for *Salmonella* sp. quantification in all of the sample types tested.
- *Campylobacter* sp. quantification with direct dilution or MPN did not work on all samples so an alternative quantitative method should be utilized.

7.2 Chapter 3 Quantification of *Campylobacter* in cattle before, during and after the slaughter process

- The overall objective of this study was to determine if there was any association with the final meat product and the other samples collected "upstream." No associations were found between the final meat product and other samples.
- The *Campylobacter* prevalence (78%) we observed in fecal samples on farm was within the range of prevalence reported by previous authors.
- We found a higher proportion (55%) of *Campylobacter* positive carcasses than has been reported in a number of previous studies, the samples for this study were obtained from a much larger surface area and were sampled before chill compared to the other studies.
- The prevalence (12%) of *Campylobacter* in the ground beef that we observed is comparable to other studies.
- The shift in *Campylobacter* species between feces (90% *C. jejuni*) to meat (100% *C. coli*) was unexpected and may represent a natural shift in the predominant *Campylobacter* species present in these different environments.

- The individual animal level may not be ideal for investigating these associations. Instead it might be more appropriate to investigate these possible associations at the group or herd level.
- There was a large reduction in prevalence and concentration of *Campylobacter sp.* as the cattle moved through the slaughter process, indicating that the process is effective in reducing *Campylobacter* sp.
- Only *Campylobacter coli* was found on the retail meat indicated that the strains recovered from the cattle on the farm and lairage were not the same as what was found on the meat.

7.3 Chapter 4 Quantification of *Campylobacter* in swine before, during and after the slaughter process

- The overall objective of this study was to see if there were any associations between contamination of the final meat product and the samples collected "upstream." We observed a positive correlation between *Campylobacter* concentrations in the farm or lairage pre-fecal samples and concentration of *Campylobacter* on ribs.
- The *Campylobacter* prevalence (90.4%) we observed in fecal samples on farm was within the range of prevalence reported by previous authors.
- The prevalence (100%) of *Campylobacter* we observed on carcasses is much greater than previously reported the samples for this study were obtained from a

much larger surface area and were sampled before chill compared to the other studies.

- The proportion (48.9%) of positive rib samples contaminated with *Campylobacter* was greater in the present study when compared to other studies conducted using raw pork, a much larger initial sample size (454 g) was obtain when compared to other studies (25 g).
- The average *Campylobacter* concentrations reported in the present study were higher than reports in the literature for some of the sample types/locations.
- There was a large reduction in prevalence and concentration of *Campylobacter sp.* as the swine moved through the slaughter process, indicating that the process is effective in reducing *Campylobacter* sp.

7.4 Chapter 5 Evaluating how quantitative PCR compares to the most probable number method or direct dilution for concentrations of *Campylobacter* in cattle and swine

- There was no association observed when the QPCR was compared to direct dilution or MPN for measuring concentrations of Campylobacter in cattle or pig samples.
- Our results indicate that quantitative culture (direct dilution and MPN) is the most appropriate means of quantifying *Campylobacter* in cattle and swine samples.

- To address the possibility of PCR inhibitors present in the sample a spiking experiment was conducted and we found that there was no significant inhibition occurring.
- The prevalence and concentration of *Campylobacter* in pigs was higher than that observed in steers.
- There was no agreement between the two methods and each of the positive samples for MPN or direct dilution were PCR confirmed thus it appears that the Q-PCR is producing false negatives suggesting inadequate sensitivity.

7.5 Chapter 6 Antimicrobial Susceptibility and Multi-Locus Sequence Typing of *Campylobacter coli* in swine before, during and after the slaughter process

- Our results indicate that clonal *C. coli* isolates can be recovered from feces, hide, carcass, and final fresh meat product of the same animal.
- This result implies that identification of effective on-farm interventions to reduce *C. coli* infection of pigs is likely to result in reduced contamination of fresh pork products.
- Five new Sequence Types (STs) were identified (ST-4083, ST-4084, ST-4085, ST-4086, and ST-4087).

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