ABSTRACT

THAKUR, SIDDHARTHA. Phenotypic and Genotypic characterization of *Campylobacter* isolated in swine from Conventional and Antimicrobial free farms. (Under the direction of Dr. Wondwossen A. Gebreyes.)

Campylobacter is the leading cause of bacterial foodborne infections in the world. *Campylobacter coli* is an important species and ranks second to *Campylobacter jejuni* in causing clinical infection in humans in the United States. Pigs have been shown to be the major reservoir of this pathogen at the pre-harvest level in conventional swine farms where antimicrobials are used for therapeutic and growth promotion purposes. Another important facet of the swine industry is the interest in the antimicrobial free (ABF) production systems. The projected increase of 20-30% in the consumption of food products reared under antimicrobial free conditions in the United States necessitates the need to determine the status of major foodborne pathogens such as *Campylobacter* in swine reared under the ABF system.

The purpose of this study was to determine the prevalence, antimicrobial resistance and the genetic diversity of *Campylobacter* isolated from swine reared in the two production systems at preharvest and during slaughter process. To better understand the epidemiology of this pathogen in the ABF system, similar comparisons were made between ABF herds reared in intensive units barns to those reared in the extensive environment. *C. coli* was the predominant species (99%) isolated from both the systems. High prevalence observed at the nursery level indicates that sows could act as an important source of transmission. The role played by environmental factors in transmission was highlighted by higher prevalence observed in ABF pigs reared outside with free access to soil and water. Significant reduction

in prevalence was seen at the slaughter level though *C. coli* were isolated from post-chill samples. We recommend adding an intervention step after chilling and before packaging of the pork product.

Highest frequency of resistance was seen against tetracycline and erythromycin in both the systems. We found significant association between antimicrobial resistance and the conventional production system. High frequency of pansusceptible isolates from the ABF system could be due to the absence of selective pressure of antimicrobials. Resistance against the fluoroquinolone ciprofloxacin and multidrug resistant *C. coli* in both systems points to the role of other unknown selective pressures and /or risk factors besides antimicrobial use. This is the first study in the US reporting ciprofloxacin resistant *C. coli* from swine. Ciprofloxacin resistance in *C. coli* is concerning as this antimicrobial is used for treating severe cases of campylobacteriosis in humans.

Multilocus sequence typing was found to be a better method compared to pulsed field gel electrophoresis for genotyping *C. coli* based on its discriminatory power and throughput. High genetic diversity of *C. coli* was observed in the swine production systems as indicated by the weak clonal structure of the isolates tested. Evidence of a lineage in the ABF system will help in comparing *C. coli* isolates from different parts of the world. Linkage equilibrium between the housekeeping genes in isolates from the two systems could explain the high antimicrobial resistance exhibited by ABF pigs irrespective of use of antimicrobials at the farm.

Overall, this study highlights the dissemination of antimicrobial resistant *C. coli* in swine irrespective of the production system. Resistance detected in the absence of antimicrobial selective pressure highlights the role played by unknown factors that need to be

determined. We recommend studies that should be focussed on finding these sources so that effective preharvest control measures could be implemented. Finally, we recommend genotyping more isolates to better understand the epidemiology of *C. coli* in the swine environment.

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *CAMPYLOBACTER* ISOLATED IN SWINE FROM CONVENTIONAL AND ANTIMICROBIAL FREE FARMS

by

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DEDICATION

To my parents

Colonel Inder Singh Thakur

Asha Thakur

PERSONEL BIOGRAPHY

Siddhartha Thakur was born on 21st February, 1974 in Maharashtra, India. He received his secondary education from Kendriya Vidyalaya, New Delhi, India. He joined the College of Veterinary Science at Gobind Ballabh Pant University of Agriculture and Technology in 1993 and graduated with degree in Bachelor of Veterinary Science and Animal Husbandry in 1998. He was awarded the Indian Council of Agriculture Research Junior Research Fellowship and joined the Indian Veterinary Research Institute in 1998. He received his postgraduate degree in Master of Veterinary Science in the department of Veterinary Public Health in 2000. In December 2001, he joined the Comparative Biomedical Sciences graduate program in the College of Veterinary Medicine at North Carolina State University where he worked on his PhD under the direction of Dr. Wondwossen Gebreyes. The major focus of his graduate work was on studying the molecular epidemiology of *Campylobacter* in swine production. He will join the Center for Veterinary Medicine at Food and Drug Administration, Laurel as a visiting scientist and work on projects in collaboration with Dr. Gebreyes food safety lab.

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1. Literature Review

1.1 Introduction and General Review

Foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations and 5000 deaths in the United States every year (Mead et al., 1999). *Campylobacter* is the leading cause of foodborne bacterial illnesses and is responsible for an estimated 2.4 million illnesses in the United States and 54,000 cases in the United Kingdom (Anonymous, 2001). The preliminary data estimated by FoodNet for diseases caused by enteric pathogens for the year 2004 shows *Campylobacter* having an incidence of 12.9 per 100,000 persons (CDC, 2005). A recent population based surveillance study conducted in the United Kingdom concluded that *Campylobacter* caused the greatest impact on the healthcare sector with 15,918 hospitalizations (Adak et al., 2005). In the US, the estimated annual cost of campylobacteriosis has been estimated to be between $ 1.3 billion and $ 6.2 billion, which increase when sequelae like the Gullian-Barre syndrome and reactive arthritis, are taken into account (Buzby et al., 1997). Assuming 55-70% of the cost is attributable to foodborne sources, the cost of foodborne campylobacteriosis range from $0.7 to $ 4.3 billion.

Theodor Escherich first described *Campylobacter* in 1886 as non-culturable spiral-shaped bacteria (Escherich, 1886). They were initially identified as *Vibrio* species and first cultured in 1913 by McFadyean and Stockman from aborted ovine feces which were later named as *Vibrio fetus* subsp. *intestinalis* (McFadyean and Stockman., 1913). Over the years, other members of the *Vibrio* group identified included *Vibrio jejuni* and *Vibrio coli* isolated from bovine and pigs, respectively (Jones and Little, 1931; Doyle, 1944). Based on the low DNA base composition and their microaerobic growth conditions, these *Vibrio* like organisms were placed in the new Genus *Campylobacter* by Sebald and Veron in 1963

(Sebald and Veron, 1963). The family *Campylobacteraceae* was finally proposed in 1991 and currently includes *Campylobacter*, *Arcobacter* and *Sulfurospirillum* (Vandamme and De Ley, 1991). There are currently 18 known species of *Campylobacter* with *Campylobacter jejuni* and *Campylobacter coli* being the two predominant species seen in humans and animals. Other important species include *Campylobacter lari*, *Campylobacter upsaliensis, Campylobacter hyointestinalis, Campylobacter fetus and Campylobacter sputorum.*

Campylobacter are Gram negative, curve shaped slender rods, 0.2 to 0.8 µm wide and 0.5 to 5µm long. They are motile and move in a typical corkscrew-like motion with the help of flagella at one or both the ends. The cells of majority of the *Campylobacter* species require microaerobic growth conditions (CO_2: 10%, O_2: 5% and N_2: 85%) and need to be incubated at 42^{o}C for 48 hours (Thompson et al., 1990). Except *C. jejuni*, all the remaining species are hippuricase negative. However, reports of hippuricase negative *C. jejuni* have been described in the literature (Totten, 1987; Rautelin et al., 1999). The phenotypic characteristics of the different species of *Campylobacter* genus including their biochemical properties and selective growth requirements are highlighted in Table1.1.

C. jejuni and *C. coli* are the two main species responsible for causing infection in humans. In humans, *C. jejuni* is responsible for causing the majority of the infections (95%) while *C. coli* are attributed for the remaining (Altekreuse et al., 1999; Gillespie et al., 2002). *Campylobacter* enteritis is characterized by diarrhea, intense abdominal pain and prostration and can range from mild enteritis to sever abdominal pain. Sequelae to the infection include autoimmune mediated demyelinating neuropathies Gullian-Barre and Miller Fisher syndromes (Nachamkin et al., 1998). Infection is mostly sporadic in nature and transmission to humans is thought to occur through consumption of contaminated food products, drinking

contaminated milk and water, and, handling of animals and pets (Sails et al., 2003; Wood et al., 1992; Yang et al., 2003; Adak et al., 1995; Damborg et al., 2004). Although antimicrobials are not recommended for treating mild campylobacteriosis cases, they are prescribed in complicated systemic cases (Allos et al., 2001; Picher et al., 1987). Resistance to important classes of antimicrobials like the fluoroquinolones and macrolides used in the treatment of severe cases of campylobacteriosis has reported to be on the rise in the US since 1990 (Allos, 2001; Picher et al., 1987). The emergence of antimicrobial resistant strains in food animals due to the use of different class of antimicrobials for therapeutic and growth promotion purpose is important to note from food safety perspective. Studies completed at the field and in the laboratories have shown the association between use of antimicrobials at farm and resistance in pathogens. The emergence and implication of fluoroquinolone resistant *Campylobacter* species especially *C. jejuni* with use of fluoroquinolone as growth promoters for the increase in the prevalence of ciprofloxacin resistant *Campylobacter* in humans is concerning (Gupta et al., 2004).

Campylobacter species were isolated from a large number of animal species and humans. Pigs and poultry are the primary reservoirs of *C. coli* and *C. jejuni,* the two important species of this pathogen from foodborne disease perspective. Colonization by *Campylobacter* in food animals has been shown to start at a very early stage in their lives. Intestinal colonization in broiler chicks starts at around 7 days of age and they remain asymptomatic carriers throughout their life (Gibbens et al., 2001). Pigs have been shown to have a high prevalence of *C. coli* and reported in up to 100% of the swine sampled (Saenz et al., 2000). Dressed pig carcasses have also been shown to have a higher prevalence of *C. coli* than either cattle or sheep (Nesbakken et al., 2003). Various animal species besides pigs and

poultry have been shown to harbor *Campylobacter* species including cats, dogs, cattle, sheep and migratory birds (Baker et al., 1999; Padungton and Kaneene, 2003; Dilworth et al., 1988; Mannering et al., 2004; Browman et al., 2004; Wilson and Moore, 1996). Numerous outbreaks have been caused by *Campylobacter* species in humans where food animals and their products have been implicated as source of infection (Smith et al., 2004; Sails et al., 2003; Hanninen et al., 2003; Ronveaux et al., 2000).

The sheer number of *Campylobacter* strains isolated in both the developed and developing countries in humans and animals makes it very important to subtype them quickly with methods that have high discriminatory power. This is important for epidemiological studies and for outbreak investigations where it is essential to pinpoint the source of infection. The use of these methods also helps in effective monitoring of strains that are temporally and spatially specific to geographic regions and also in developing prevention strategies for controlling the pathogen. Numerous phenotypic and genotypic methods are available to type *Campylobacter*. The phenotypic methods include antimicrobial sensitivity testing using agar dilution method (Ge et al., 2002), epsilometric test (E-test) (Ge et al., 2002), disk diffusion test (Leatherbarrow et al., 2004), serotyping based on heat stable (Penner and Henessy, 1980) and heat labile (Lior, 1994) antigens, phage typing (Salama et al., 1990) and biotyping (Bolton et al., 1984). The phenotypic methods used are generally quick and cheap but are not as discriminatory as the genotypic methods. A good review of the different genotypic methods used for subtyping *Campylobacter* is available in the literature (Wassenaar and Newell, 2000). Genotyping methods commonly used include pulsed field gel electrophoresis (PFGE) (Ribot et al., 2001), multilocus sequence typing (MLST) (Dingle et al., 2001), rapid amplification of polymorphic DNA (RAPD) (Wassenaar

and Newell, 2000), flagellin-A gene- restriction fragment length polymorphism typing (*flaA*-RFLP) (Fitzgerald et al., 2001) and amplified length polymorphism (AFLP) (Schouls et al., 2003) to name a few. A combination of both the phenotypic and genotypic methods has been recommended for epidemiological studies (Patton et al., 1991).

The predominance of *C. jejuni* in human infections (> 95% of human cases) has led to ignorance of the impact of less prevalent species like *C. coli*. However, recent studies done in Spain and UK have highlighted the importance of *C. coli* as an important human pathogen due to it ability to show resistance to various classes of antimicrobials and in causing more indigenously acquired foodborne diseases (Saenz et al., 2000). The importance of this species has further been illustrated by a recent study conducted in the UK where it was found to be 3.5 times more responsible for causing indigenously acquired foodborne diseases than *Salmonella* Typhimurium (Tam et al., 2003).

There is paucity of information on the comparative significance of *Campylobacter* isolated from pigs reared in antimicrobial free (ABF) and conventional production systems. The status of *Campylobacter* in swine raised in the conventional system of production where antimicrobials are used both for treatment and growth promotion has been reported previously in many studies (Van Looveren et al., 2004; Payot et al., 2004; Saenz et al., 2000). In the US, food animal production that does not use any antimicrobials during the production phase (organic farming) is on the rise accounting for approximately 1-2% of the total food sales. The consumption of food products grown under this category is expected to increase by 20-30% annually (APHIS, 1997). Studies looking at the prevalence and antimicrobial resistance profile of *Campylobacter* in the two distinct production systems have been reported before in other host species such as poultry and dairy cows in the United States

(Heuer et al., 2001; Sato et al., 2004). Besides looking at the prevalence and antimicrobial resistance, it is also important to study the genetic diversity/similarity of this pathogen in the two production systems to determine whether the same or distinct clones exist in them. To address the above points, the present study was conducted to determine and compare the prevalence, antimicrobial susceptibility and the genetic diversity of *Campylobacter* isolated from swine reared in the conventional and ABF production systems and at the pre-harvest production level including farm and slaughter.

1.2 *Campylobacter* prevalence in Swine at farm and slaughter

Pigs are the primary reservoirs for *C. coli* (Harvey et al., 1999; Payot et al., 2004). A high prevalence of this species in pigs ranging from 46% to 100% has been reported (Nielsen et al., 1997; Saenz et al., 2000). Newborn piglets can get infected with *Campylobacter* within 24 hours of birth indicating the farrowing units to be the primary site of horizontal transmission. Although vertical transmission of *Campylobacter* from infected sows to unborn piglets has not been demonstrated so far, the high prevalence rates observed in newborn piglets does support this hypothesis. Young et al., reported prevalence of *Campylobacter* ranging from 57.8% for newborn piglets to 100% for weaned piglets (Young et al., 2000). In another study conducted in sows and their young piglets at 21 days of weaning, *C. coli* was isolated from 100% of the fecal and rectal swabs collected (Hume et al., 2002). Similarly, Weijtens et al. reported pigs with intestinal *Campylobacter* at 11 weeks of age and remaining positive for this pathogen until slaughter (Weijtens et al., 1993). Newborn piglets and pigs at weaning have been shown to be positive for *Campylobacter* if they are constantly exposed to infected feces through infected sows (Harvey et al., 2000). In that study, none of the newborn

piglets separated from the sows after birth had *Campylobacter* as compared to the sows-reared piglets that were 100% positive after 20 days. All these studies point to the high susceptibility of newborn piglets becoming colonized by *Campylobacter*.

Campylobacter spp. has been reported for market age pigs in finishing farms and at slaughter houses with prevalences ranging from 50.4% to 100% (Payot et al., 2004; Pezzotti et al., 2003; Young et al., 2000; Harvey et al., 1999; Saenz et al., 2000). *C. coli* was the predominant species isolated in most of the studies with prevalence ranging from 60% to 100% (Harvey et al., 1999; Saenz et al., 2000). Although *C. jejuni* and *C. lari* are primarily isolated from poultry and water fowl, high prevalence of these species has been reported from 76% and 4% of the pigs, respectively (Harvey et al., 1999). *C. jejuni* has also been isolated from 76.3% and 83% of gilts and pregnant sows and in the cecal content of 2.3% of the pigs at the slaughter house (Young et al., 2000; Boes et al., 2005). A recent survey conducted in food animals from four European Union countries reported *Campylobacter* prevalence in pigs ranging from 35% to 50% with *C. coli* being the predominant species identified in 71% of the pigs followed by *C. jejuni* in 21% (Bywater et al., 2004).

Isolation of *Campylobacter* from swine carcasses in the literature has varied from 2% in Belgium to 95% in Sweden (Korsak et al., 1998; Kwiatek et al., 1990; Svedhem and Kaijser, 1981). Environmental contamination of the carcasses at the slaughter plant has been reported before with *Campylobacter* isolated from the workers gloves, gambrel table and the dehairer had the same fingerprint as *C. coli* and *C. jejuni* isolates from the swine carcasses indicating cross contamination (Cloak and Fratamico, 2002). Another important source of carcass contamination includes the animal itself via bacteria through the pharynx and the feces. The Food Safety Inspection Service (FSIS) of the United States Department of

Agriculture (USDA) ensures the meat product safety through the Hazard Analysis Critical Control Point (HACCP) pathogen reduction program. The Nationwide Pork Microbiological Baseline Data Collection Program was designed by FSIS to safeguard the safety of pork products and also monitor the prevalence of important foodborne pathogens of public health concern including *C. jejuni, C. coli, Salmonella, Escherichia coli O157:H7, Clostridium perfringens, Staphylococcus aureus* and *Listeria monocytogenes* in different slaughter houses across the United States (FSIS, 1996). This program is designed to monitor 99.5% of all the market hogs slaughtered and 94% of all the swine slaughtered under Federal inspection. According to the report from FSIS, *C. jejuni* and *C. coli* were recovered from 31.5% of the 2112 carcasses swabbed (FSIS, 1996). Both *Campylobacter* species had the highest prevalence among the pathogenic species of bacteria being monitored under this program. Previous studies have also reported their presence on swine carcasses in the slaughterhouse at different stages of processing ranging from 2% to 9% at pre-chilling to 1.7% at the post-chilling stage (Korsak et al., 1998; Nesbakken et al., 2003; Oosterom et al., 1985).

There are many studies that have been conducted in the past reporting the prevalence of *Campylobacter* on swine carcass before chilling and after chilling with significant reduction ranging from 13% at pre-chilling to 0% at post-chilling (Pearce et al., 2003; Oosterom et al., 1985; Bracewell et al., 1985). *Campylobacter* is highly susceptible to cold and drying conditions and studies have reported significant reduction of this pathogen on post-chill samples (Chang et al., 2003; Oosterom et al., 1985). In the experimental study conducted by Chang et al., the authors reported blast chilling method to be more effective than the conventional chilling method to significantly reduce *C. coli*, *Salmonella* Typhimurium and *Listeria monocytogenes* on pork carcasses.

Food contamination by important foodborne pathogens including *Campylobacter* can take place at multiple critical points steps during the production stages to the retail market. Epidemiological studies have implicated food of animal origin as the major vehicles for the transmission of foodborne pathogens (Gupta et al., 2004). *Campylobacter* species including *C. coli* have also been isolated from retail pork products from 1.3-1.7% of the samples tested in the United States, 2% in Belgium, 5% in Austria, 10.3% in Italy and 16.9% in Canada (Duffy et al., 2001; Zhao et al., 2001; Korsak et al., 1998; Mayrhofer et al., 2004; Pezzotti et al., 2003; Hariharan et al., 1990). Zhao et al also reported the presence of multiple *Campylobacter* species present in raw meats indicating that meat products may be contaminated by more than one strain (Zhao et al., 2001). External packaging of retail pork has also been found to be contaminated (0.2%) by *Campylobacter* and could be a possible source of cross contamination after buying these meat products (Burgess et al., 2005).

1.3 *Campylobacter* prevalence in food animals reared in the ABF production system

Limited information is available in the literature on the prevalence of *Campylobacter* in food animals that are reared under production systems where no antimicrobials are added either for growth promotion and therapeutic purposes. Few such studies have been done in poultry and cattle. Although ABF free and organic pork products are available for retail, there is no information on the prevalence of *Campylobacter* in these swine. Heuer et al. compared the prevalence and antimicrobial resistance of *Campylobacter* in broiler flocks reared in organic (antimicrobial free), conventional and extensive indoor production farms. The prevalence ranged from 36.7% in conventional broilers to 100% in organic broilers and 49.2% in the extensive indoor broiler flocks (Heuer et al., 2001). Organic broiler flocks had

significantly higher *Campylobacter* positive flocks than those from the other two production systems. The authors reported that organic broiler production constituted a strong potential for introduction of *Campylobacter* at slaughter due to their different rearing conditions. Among them, exposure to the outside environment including soil, water, animals and the higher age at slaughter were the two important factors. Avrain et al., also reported a higher prevalence of *Campylobacter* (80%) in free range broilers compared to 56.6% in standard and 51.3% in export quality broilers (Avrain et al., 1997). Another study conducted in France compared *Campylobacter* isolated from broilers before and after the antimicrobial growth promoter ban and reported high prevalence in all the studied flocks (Desmonts et al., 2004).

Sato et al. conducted a similar study in organic and conventional dairy herds looking at comparing the prevalence and antimicrobial resistance of *Campylobacter.* They reported a prevalence of 26.7% and 29.1% in organic and conventional farms, respectively, and the prevalence was not statistically different between the two production systems (Sato et al., 2004). A recent study conducted in the US compared FQ resistant *Campylobacter* isolates from conventional and antibiotic free chicken products (Price et al., 2005). FQ resistant *Campylobacter* was isolated from both the production systems indicating that FQ resistance may persist even in the absence of FQ selective pressure. These studies indicate that prevalence of *Campylobacter* is high in animals reared in the ABF system.

1.4 Antimicrobial use in Food animals

The discovery of antimicrobials in the early part of the 20th century greatly reduced the threat posed by infectious agents. Over the years, the use of antimicrobials has saved the lives of millions of people all over the world. The first ever antimicrobial discovered was

pyocyanase in 1888 by German microbiologist E. de. Freudenreich. However, it was not used since it was highly toxic to patients and unstable. The antimicrobial era began in 1910 with the use of the drug Salvarsan in treating cases of syphilis followed by the discovery of sulfonamides in 1930s. The use of antimicrobials for treating clinical cases began in 1942 with the discovery of the first antibiotic penicillin by Alexander Fleming in 1928 (Fleming, A., 1929). The success of this wonder drug in treating patients and the concurrent discovery of other antimicrobials led to the rapid increase in the production of antimicrobials. Antimicrobials were now being administered parenterally, orally and topically in humans and animals to treat and prevent diseases.

The growth promoting properties of antimicrobials in food animals was also observed around the same time in 1940s when chicken fed with fermentation waste of tetracycline grew faster than those that were not fed with it (Stokstad et al., 1949). The practice of feeding sub therapeutic concentration of these antimicrobials for growth promotion in animals became an integral part of the livestock production over the decades since its first use. At least 23 products with antimicrobial activity in the year 2001 were approved by the Food and Drug administration (FDA) for use as feed additive applications. Currently, more than 40% of the antimicrobial production is being used in animals for growth promotion purposes (Institute of Medicine Workshop Report, 1998). The Animal Health Institute (AHI) in the United Studies estimated approximately 18 million pounds of these antimicrobials used for growth promotion in animals in 1999 (AHI, 2000). However, the Union of Concerned Scientists reported an estimated 25 million out of the 29 million antimicrobials produced annually in the US in 2001 used for growth promotion in animals (Mellon et al., 2001).

Antimicrobial growth promoters (AGP) are used in the swine industry at the nursery and the finisher farms in feed. AGP are added in the feed of weaner pigs as starter rations since the pigs are most susceptible to infection at this stage. Antimicrobials are subsequently removed from the pig feed toward the end of the finishing phase to avoid drug residues. The different classes of antimicrobials used for therapeutic and growth promotions in the swine industry are highlighted in Tables 1.2-1.7. The commonly used antimicrobials in swine include tetracycline, tylosin and sulfamethazine (McEwen and Fedorka-Cray, 2002). Different antimicrobials used for growth promotion and treatment include ceftiofur, sulfonamides and tetracycline for preventing pneumonia, gentamicin, apramycin and neomycin for treating bacterial diarrhea and lincomycin, tiamulin and macrolides for treating swine dysentery (Dunlop et al., 1998). AGP added in the feed of pigs has been shown to increase the efficiency of feed conversion by 3-4% and improve the live weight gain by 5-6%, especially in the young pigs (Armstrong, 1986). Antimicrobials like ceftiofur, sulfonamides and tetracycline are used to treat and prevent pneumonia; lincomycin, tiamulin and macrolides are used to treat swine dysentery and ileitis; and gentamicin and apramycin are used to treat bacterial diarrhea caused by *E. coli* and *C. perfringens* (Dunlop et al., 1998; McEwen and Fedorka-Cray, 2002).

1.4.1 Implications of Antimicrobials use in the food industry

There are benefits of using AGP in animals. Many studies have been done showing growth promotion and increased feed efficiency in animals (Lawrence, 1998). The exact mechanisms by which the AGP elicit their beneficial effect are not clearly known. There are numerous mechanisms that have been hypothesized including; 1) reduction in the harmful

pathogens in the gut that reduce growth and cause sub-clinical infection, 2) reduced production of growth suppressing toxins and metabolites, 3) effective absorption of the nutrients and 4) availability of more nutrients to the host due to reduction in the gut microflora (Knudsen, 2001).

The use of antimicrobials, however, creates a selective pressure on the pathogen to either adapt to that particular antimicrobial or perish. This has subsequently led to the development of resistance in these pathogens against antimicrobials (Anderson et al., 2003). Except a few antimicrobials including bambermycins, bacitracin and carbadox, most of the products used in animals for treatment and growth promotion are often closely related to antimicrobials used in humans. For example, tetracycline and macrolides are used for stabilizing the pig gut flora at the time of weaning. These drugs are also used for treatment purposes in humans. Therefore, pathogens that become resistant in animals to any class of antimicrobial can show cross-resistance to another antimicrobial of the same class. *Campylobacter* isolates resistant to ciprofloxacin are also cross resistant to nalidixic acid as the resistance mechanism involving point mutation in the quinolone resistance determining region (QRDR) of the *gyrA* gene is the same for both these fluoroquinolone antimicrobials (Anderson et al., 2003). There are studies highlighting the transfer of antimicrobial resistant bacteria including *Campylobacter* and *Salmonella* from food animals to humans through the food chain (Altekreuse et al., 1999; Angulo et al., 2000). The Alliance of Prudent Use of Antibiotics (APUA) and the Institute of Medicine have also reached similar conclusions in the US (APUA, 2002; Fey et al., 2000; Angulo et al., 2000; AHI, 2000). However, it is difficult to show exactly how the individual was infected with resistant pathogen. The route

of transmission of an antimicrobial resistant strain from the source to humans or vice-versa is very complex.

The emergence of fluoroquinolone (FQ) resistance in humans is a good example of the development of antimicrobial resistant development in pathogens in animals and their subsequent transfer to humans. FQ were licensed for use in the humans in 1986 and in poultry industry in 1996. Surveys done in 1989 and 1990 did not find any *Campylobacter* resistant to FQ. Nalidixic acid resistant strains first started appearing in 1992 (1%) to up to 10% of the total isolates in 1998. Genotyping of human and retail poultry strains in Minnesota showed significant association between FQ resistant *C. jejuni* and domestically acquired infections (Smith et al., 1999). In the US survey conducted by FoodNet, 58% of the FQ infections are indigenously acquired due to consumption of poultry (Gupta et al., 2004). Eventhough it is difficult to prove conclusively that this is really the case; it does portray an interesting picture. Experimental studies done in pigs have shown to covert FQ susceptible strains of *C. coli* to FQ resistant strains after a single dose of enrofloxacin (Delsol et al., 2004). However, there are other studies that failed to show this kind of association between use of antimicrobial and development of resistance (Wells et al., 2001; Dargatz et al., 2000).

Banning the sub-therapeutic use of antimicrobials in feed has been shown to be accompanied by the decrease in resistance to these drugs. Sweden and Denmark have banned the use of most of the antimicrobials. In fact, Sweden has banned the use of any antimicrobial for growth promotion in animals since 1986 (Wierup et al., 1987). In Denmark poultry industry, there was decrease of < 1% in feed efficiency after antimicrobial withdrawal. There was an increase in necrotic enteritis but no increase in the death rate (Emborg et al., 2001). Decrease in Virginiamycin and avilamycin were accompanied by decrease in pathogens

resistant to these drugs while *Lawsonia intercellularis*, which infects pigs, has become a problem (McEwen and Fedorka-Cray, 2002).

Evans and Wegener reported in 2003 that the ban on antimicrobials in animal feed has led to a significant decrease in *Salmonella* in broilers, swine, pork and chicken meat (Evans and Wegener, 2003). The prevalence of macrolide resistant *C. coli* in swine has declined after the ban (Danish Veterinary Institute, 2003). However, there was no change in the prevalence of *Campylobacter* in broilers. A recent WHO panel made to review the effect of banning growth promoting substances in Denmark reported that the primary objective to reducing the resistance gene pool has been achieved and that other countries would benefit in the same way as Denmark (WHO, 2003). The economic impact of banning AGP in the US swine industry was calculated in a study that was based on the Swedish pork industry (Hayes et al., 1999). The initial production cost would increase by $6.05 per animal that was projected to drop down to $5.24 per animal after 10 years. The anticipated loss of feed efficiency and piglet loss was expected to increase the cost of retail pork by $0.05 per pound.

1.5 Antimicrobial resistance in *Campylobacter*

Resistance in *Campylobacter* against antimicrobials has been reported from humans and animals from different parts of the world. Overall, *C. coli* has been shown to be resistant to a larger number of antimicrobials than *C. jejuni* (Aaresterup and Engberg, 2001; Saenz et al., 2000; Pezzotti et al., 2003). Resistance has been shown in both *C. jejuni* and *C. coli* against FQs, macrolides, tetracycline, β lactams, aminoglycosides and chloramphenicol. FQ and ampicillin resistance has been seen in *C. coli* ranging from 15% to 100% and 20% to 66%, respectively (Payot et al., 2004; Saenz et al., 2000). FQ resistance is commonly seen in

C. jejuni isolated from poultry and humans (Saenz et al., 2000; Bywater et al., 2004; Smith et al., 1999). The prevalence of ciprofloxacin resistant *Campylobacter* in humans in the US has increased significantly from 13% to 19% from 1997 to 2001 (Gupta et al., 2004). High frequency of ciprofloxacin resistance was observed in 99% of *Campylobacter* isolated from poultry, 35% from retail chicken and 0.6% from cattle (Saenz et at., 2000; Sato et al., 2004). Saenz et al. reported 100% resistance against ciprofloxacin, nalidixic acid and cephalothin in *C. coli* isolated from pigs in Spain (Saenz et al., 2000). In that study, high frequency of resistance was also observed against azithromycin (81%), spiramycin (81%), virginiamycin (94%), and ampicillin (65.7%).

High resistance against erythromycin and tetracycline in pigs has been reported before with *C. coli* being significantly more resistant to them than *C. jejuni* (Van Looveren et al., 2001; Pezzotti et al., 2003). Resistance to tetracycline has been observed ranging from 76.6% to 94.4% and against erythromycin in 42.6% to 81.4% of *C. coli* isolated at farm (Pezzotti et al., 2003; Saenz et al., 2000). Tetracycline resistance frequency is one of the highest seen in *Campylobacter spp.* from 13.6% to 46% in humans (Aquino et al., 2002; Hakanen et al., 2003), 32% to 58.4% in poultry (Saenz et al., 2000; Bywater et al., 2004), and 82% in retail meats (Ge et al., 2003). Higher frequency of resistance against the macrolide erythromycin in humans has been observed more commonly in *C. coli* (0% to 68.4%) than *C. jejuni* (0% to 11%) isolates (Engberg et al., 2001). Resistance against erythromycin has also been reported in *Campylobacter* from poultry (61.7%), retail meats (17%), and cattle (5.6%) (Desmonts et al., 2004; Saenz et al., 2000; Bywater et al., 2004). Hart et al. isolated *Campylobacter* from pig carcasses at pre-evisceration and pre-chiller stages and reported high resistance against erythromycin, lincomycin, tetracycline and tylosin (Hart et al., 2004).

In a recent study comparing *Campylobacter* in conventional and organic retail meat from poultry, higher percentage of samples from the organic meat were contaminated with *Campylobacter spp.* having a higher rate of resistance against erythromycin and tetracycline (Meng et al., 2005).

Resistance against gentamicin and chloramphenicol has been observed in low frequencies in humans and animals. Gentamicin resistance has been observed in 0.5% to 1.8% of *Campylobacter* isolated from chicken, 0.7% to 3.7% in cattle, 1.4% from chicken meat and 1.6% humans (Bywater et al., 2004; Van Looveren et al., 2001; Pezzotti et al., 2003; Hakanen et al., 2003). Low level of resistance has been seen against gentamicin (3.3%) in pigs (Van Looveren et al., 2001). Chloramphenicol resistance in *Campylobacter* has been reported in 2.7% of the isolates in humans and in 0.4% case of isolates from poultry (Hakanen et al., 2003; Oza et al., 2003).

Multidrug resistant (MDR) isolates of *Campylobacter* showing resistance to three or more antimicrobials simultaneously have been reported from pigs and poultry in 3.3% of *C. jejuni* and 3.8% of *C. coli* isolates (Randall et al., 2003). Hakanen et al. reported 22% of the total *C. jejuni* strains to be MDR that were significantly associated with ciprofloxacin resistance in humans (Hakanen et al., 2003). *C. coli* (17%) isolated from food animals including broilers, pigs and turkey has been shown to me MDR in higher frequency than *C. jejuni* (7.6%) (Van Looveren et al., 2001). Payot et al., reported 37% of *C. coli* isolated from pigs as MDR with erythromycin nalidixic acid and tetracycline pattern being the most common in 77% of these MDR strains (Payot et al., 2004).

1.5.1 Mechanism of antimicrobial resistance in *Campylobacter*

The resistance mechanisms against different antimicrobials observed in *Campylobacter* are highlighted in Table 1.8. Resistance against FQ has been attributed to point mutations in the quinolone resistance determining region (QRDR) of *gyrA* of this pathogen that include the Type II topoisomerases. The most common mutation in the QRDR region is in the amino acid position 86 when threonine (Thr) substituted by Isoleucine (Ile) (Ruiz et al., 1998). Other mutations in the QRDR region have also been detected including Asp-90-Asn change, Thr-86-Lys change and mutations in *parC* region (Luo et al., 2003; Gibreel et al., 1998). Recently, interaction of plasmid mediated quinolone resistance protein Qnr has been shown to interact with DNA gyrase leading to resistance against the quinolones (Tran et al., 2005). Efflux pumps including the CmeABC pump have been shown to be associated with increased to resistance to FQs as well as to other antimicrobials in *Campylobacter* (Luo et al., 2003).

Resistance to the important macrolide erythromycin is elucidated via mutational changes in the 23S rRNA at positions 2074 and 2075 where the antibiotics bind at the 50S ribosomal unit (Trieber and Taylor, 2001). High level of tetracycline resistance is usually conferred by the *Tet (O)* gene located on a transmissible plasmid in both *C. jejuni and C. coli* (Taylor and Courvalin, 1988). The Tet (O) protein encoded by this gene protects the ribosome from the inhibitory effect of this antimicrobial (Connell et al., 2003). Gentamicin resistance is mediated by the chemical inactivation of the compound by *aph (3')-Ia* Gene (Aaresterup and Engberg, 2001). Resistance is due to acetylation by the enzyme chloramphenicol acetyltransferase (*cat*). In *C. coli*, a single *cat* gene has been identified so far (Wang and Taylor, 1990).

1.6 Subtyping of *Campylobacter*

1.6.1 *Campylobacter* Phenotyping

The phenotypic methods commonly used include serotyping and phage typing (Penner and Hennessey, 1980; Lior et al., 1982; Grajewski et al., 1985). Serotyping is based on detecting heat-labile (HL) antigens (Lior system) or the O antigens (heat-stable; Penner system) with the later method being the most commonly used method of serotyping. The Lior system is based on the principle of direct agglutination where traditional method of bacterial cell agglutination is used to identify the different HL antigens. The Penner system is based on the passive haemagglutination (PHA) principle (Moran and Penner, 1999). To date there are 66 serotypes identified in *Campylobacter* using the Penner system. Certain serotypes of *C. jejuni* have been identified to be associated with Gullian-Barre syndrome including HS serotype O19 (Kuroki et al., 1991). Nontypeability of strains has been a problem with serotyping. One study reported 19% of the human strains to be nontypeability including 19.4% *C. jejuni* and 12.1% *C. coli* isolates (Frost et al., 1998). Other issues with serotyping include the high cost of the reagents and the level of expertise required to correctly serotype the strain.

Phage typing has been used for further subtyping *Campylobacter* and in most cases as an extension to serotyping. Different schemes are present for phage typing with 76 phage types identified so far. In the study done by Frost et al., the authors identified 57 phage types among the 2407 *C. jejuni* isolates with 60% of the strains assigned to the 10 most common types (Frost et al., 1999). However, resistance to bacteriophages was observed recently in broiler chicken that may affect the ability to phage type these strains (Connerton et al., 2004). Similar to serotyping, phage typing also has problems of non-typeable strains and requires

the infrastructure, expensive reagents, time and expertise making it a difficult scheme to be carried out in many laboratories around the world. Because of all the above difficulties experienced with these two methods, serotyping and phage typing have limited national and international use for studying the epidemiology of *Campylobacter*. As a result, genotypic methods have been developed and have become more popular because of their availability ability to quickly exchange data between different laboratories quickly through the internet.

1.6.2 *Campylobacter* Genotyping

There are several important criteria that should be used before deciding to use a genotypic method for typing a pathogen. These include the discriminatory power of the method, typeability, reproducibility and ease of data interpretation. Genotyping method that best fits these criteria should be used. Besides following the above criteria, the bacterial genome under study should also be taken into account. *C. jejuni* has a circular chromosome of 1.6 MBp with low G+C content of 30.6% which is predicted to encode 1,654 proteins and 54 stable RNA sequences (Parkhill et al., 2000). One of the most striking findings in the genome is the presence of hypervariable sequences. Most of these hypervariable sequences are associated with genes responsible for lipopolysaccharide generation and flagellar modification. It is thought that this variation is important for the survival and dynamic colonization of the intestine by this pathogen (Parkhill et al., 2000). It is important to keep the hypervariable region of *Campylobacter* in mind before deciding to use a genotyping method. There are numerous methods used for genotyping *Campylobacter* species including multilocus enzyme electrophoresis (MEE), PFGE, MLST, ribotyping, AFLP and *flaA*-RFLP (Wassenaar and Newell, 2000). The different genotypic methods have been used for

investigating outbreaks in humans and also for the surveillance of this pathogen in animals and environmental sources. These methods have revealed the genetic diverse nature of *Campylobacter* genome. This is mainly due to the presence of the above mentioned hypervariable regions scattered throughout the *Campylobacter* genome.

The above methods have shortcomings in regard to genotyping *Campylobacter.* Many studies reported both intragenomic and intergenomic recombinations taking place in the genome of this pathogen. Harrington et al. reported both types of recombinations taking place in the flagellin subunit coding genes, *flaA* and *flab* (Harrington et al., 1997). This study had two implications. First, recombination at the flagella level helps the pathogen to generate antigenic diversity thereby helping the pathogen to escape the immunological response of the host. The second implication was that *flaA*-RFLP is not a good method for genotyping this pathogen as the restriction patterns developed will show variations over time. Ribotyping is another technique that has been beneficial in cases where species identification of *Campylobacter* has been difficult using the available phenotypic methods (Wassenaar and Newell, 2000). However, the discriminatory power of this method is not ideal and it cannot at times distinguish between some species. It has been used however for developing a primary library typing method to identify *C. jejuni* and *C. coli* (Manfreda et al., 2003).

1.6.2.1 Amplified Fragment Length Polymorphism

AFLP is based on the selective amplification of restriction fragments generated from the total genomic DNA. Selective amplification is governed by use of specific restriction enzymes and the amplification primers. This method has been used for genotyping bacterial pathogens including *Salmonella* and *Campylobacter* (Duim et al., 1999., Gebreyes and

Altier, 2003). The data generated can be digitalized and exchanged for interlaboratory comparison of fragments. AFLP has been used in many studies to determine the epidemiology of this pathogen in human gastroenteritis cases, food and animal sources (Siemer et al., 2005; Duim et al., 2003; Duim et al., 1999). Based on the AFLP profile of 174 *C. coli* isolates in the study done by Siemer et al., the authors observed that isolates from poultry rather than pigs were more closely related to the human isolates (Siemer et al., 2005). AFLP is a good method for typing but interlaboratory comparison is problematic due to the complex banding patterns observed and it is prone to variation.

1.6.2.2 Pulsed Field Gel Electrophoresis

PFGE has been used successfully for typing *C. jejuni* isolated from different outbreaks (Sails et al., 2003; Fitzgerald et al., 2001). This method is also used by PulseNet within the United States for nationwide surveillance of *Campylobacter* and other important food borne pathogens. In the study by Sails et al., PFGE was able to temporally relate outbreak strains from 10 out of the 12 outbreaks (Sails et al., 2003). However, the authors also mentioned that inability of the restriction enzyme *SalI* to cut the DNA. The presence of DNA that is refractory to cutting by the restriction enzymes is one of the drawbacks of using this method. Also, a similar problem previously had been highlighted in another study which reduces the sensitivity of this method (Newell et al., 2000). PFGE has been shown to have higher discriminatory power than MLST, *flaA* short variable region sequencing, and multilocus enzyme electrophoresis (MLEE) methods (Sails et al., 2003; Sails et al., 2003a). In another study done by Nadeau et al. in Canada, an association between the *in vitro* virulence properties and PFGE profiles was shown indicating the presence of clonally related

isolates with potential virulence properties (Nadeau et al., 2003). Another study involving 80 unrelated isolates of *C. jejuni* from humans, poultry and cattle using six different genotyping methods revealed PFGE to be one of the most discriminatory method (Nielsen et al., 2000).

Despite the proven utility of this method, there are difficulties of nontypeable *Campylobacter* strains, differences in electrophoretic conditions leading to variation in results, labor intensive instruments and expensive instruments and software required to analyze the results. Genetic variation seen in *Campylobacter* strains is another concern when using PFGE for typing. On et al. showed clearly the genomic variation in *C. coli* on subculturing the same set of six isolates from 50 times with PFGE profiles being generated after 1, 20, 40 and 50 passages (On et al., 1998). Significant alterations were seen in the profiles between PFGE runs. Spontaneous intramolecular genomic rearrangements were considered the most likely mechanism for the changes observed in this study. This study indicates that PFGE profiles may not provide stable fingerprints. The chromosomal rearrangements may lead to change in the profile thereby making it an unsuitable method for long-term study of the epidemiology of this pathogen (Hanninen et al., 1999; Wassenaar et al., 1998).

1.6.2.3 Multi Locus Sequence Typing

MLST is a suitable technique for bacterial populations that are weakly clonal in nature like *Campylobacter* (Dingle et al., 2001). This method indexes the neutral variation seen in the housekeeping genes and is analogous to MLEE. The housekeeping genes evolve slowly and are stabilized for conserving the important metabolic proteins that they code. Data generated by this method is relatively accurate since it involves comparing nucleotides of

specific sequence and size. The data can be easily reproduced and transferred via the internet for interlaboratory comparisons to study the global epidemiology of *Campylobacter* (Dingle et al., 2001; Dingle et al., 2002). This method was developed for the first time for *Neisseria meningitides* and then extended to other pathogens (Maiden et al., 1998). It has been used successfully for characterizing several bacterial pathogens including *Streptococcus pneumoniae, Staphylococcus aureus* and *Neisseria meningitides* to name a few (Enright and Spratt, 1998; Enright et al., 2000; Holmes et al., 1999).

MLST has been used to investigate outbreak investigations involving *C. jejuni*, understand the genetic diversity from farm animals and their environment and also in understanding the population structure of this pathogen (Sails et al., 2003; Colles et al., 2003; Dingle et al., 2001). The usefulness of this method to differentiate between outbreak and the endemic strains of *C. jejuni* has been shown (Sails et al. 2003). Although PFGE in this report had the highest discriminatory power, the combined approach of MLST and *flaA*-SVR typing provided the same level of discrimination as PFGE (Sails et al., 2003). MLST has been used to define the clonal complexes in the *C. jejuni* population. So far, a total of 17 *C. jejuni* clonal complexes have been defined with complex ST-21 and 45 being the largest (Dingle et al., 2002). The assignment of an isolate to one of these 17 clonal complexes tremendously helps in comparing isolates from different sources and from all over the world. In one study involving 814 *C. jejuni* isolates from humans, food animals including chicken, lamb, beef cattle and environmental sample including sand from the beaches helped group 748 (92%) of the isolates into one of the 17 clonal complexes with six of them containing 63% of the human isolates (Dingle et al., 2002).

The MLST scheme has recently been extended to *C.coli* (Dingle et al., 2005). Phylogenetic analysis of *C. jejuni* and *C. coli* using the MLST data has revealed the weak clonal structure of these two species (Dingle et al., 2005; Dingle et al., 2001). The two species share 86.5% similarity at the nucleotide level for the housekeeping genes sequenced. Dingle et al. showed evidence for genetic exchange taking place between the two species at the housekeeping gene level (Dingle et al., 2005). The study done by Manning et al. involving 266 *C. jejuni* isolates from human and animal sources showed overlapping between these isolates from different sources (Manning et al., 2003). The authors reported association between source and sequence type indicating the potential for host adaptation in this species. Duim et al. used MLST, PFGE, ribotyping and AFLP to characterize *C. jejuni* isolates from humans cases of gastroenteritis and Gullian-Barre syndrome and reported all the methods to have the same value for epidemiological typing (Duim et al., 2003). However, they pointed out the advantages MLST had over other techniques including interlaboratory portability, reproducibility and a unified nomenclature used throughout the world. A recent report identified two clonal complexes in *C. coli* consisting of isolates that were isolated from different sources including humans, chickens, swine and sheep (Miller et al., 2005).

The importance of *Campylobacter* as a leading foodborne pathogen is exemplified by the number of illnesses it causes in humans. It is important to determine the status of this pathogen in swine reared in ABF farms and compare it to those from the conventional farms both at the phenotypic and the genotypic level. This will help us in better understanding the epidemiology of this pathogen in swine production systems.

1.7 Objectives of the study

1. To determine and compare the prevalence and antimicrobial resistance profile of *Campylobacter* isolated from swine at different stages of production in the Conventional and Antimicrobial free farms (ABF) at farm and slaughter.
2. To evaluate the discriminatory power and compare Multi Locus Sequence Typing (MLST) and Pulsed Field Gel Electrophoresis (PFGE) methods for genotyping *Campylobacter* species.
3. To assess the molecular epidemiology and phylogenetic analysis of *Campylobacter* isolated from the two production systems at farm and slaughter.

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Table 1.1 Phenotypic and biochemical properties of different species of *Campylobacter* (Reference: Nachamkin, I., and M. J. Blaser. *Campylobacter* 2nd Edition: ASM Press, Washington, D. C).

Species	Alpha-hemolysis	Catalase	Hippurate - hydrolysis	Urease	Nitrate-reduction	H2S / TSI	Indoxyl acetate hydrolysis	25 °C	42 °C	Growth			
										McConkey	Glycine (1%)	Nacl (4%)	Cefoperazone (64 mg/L)
C. coli	V	+	-	-	+	V	+	-	+	V	+	-	+
C. conscious	V	-	-	-	V	-	-	-	V	-	V	-	
C. curvus	V	-	V	-	+	V	V	-	V	V	+	-	V
C. fetus subsp. fetus	-	+	-	-	+	-	-	+	V	V	+	-	+
C. fetus subsp. venerealis	V	V	-	-	+	-	-	+	-	V	-	-	-
C. gracilis	-	V	-	-	V	-	V	-	V	V	+	-	-
C. helviticus	+	-	-	-	+	-	+	-	+	-	V	-	V
C. hyointestinalis subsp. hyointestinalis	V	+	-	-	+	+	-	V	+	V	+	-	-
C. hyointestinalis subsp. lawsonii	V	+	-	+	+	+	-	-	+	V	V	-	V
C. jejuni subsp. doylei	+	V	+	-	-	-	+	-	-	-	V	-	-
C. jejuni subsp. jejuni	+	+	+	-	+	-	+	-	+	-	+	-	+
C. lari	V	+	-	V	+	-	-	-	+	-	+	-	+
C. muscosalis	-	-	-	-	-	+	-	-	+	V	V	-	V
C. rectus	+	V	-	-	+		+	-	V	-	+	-	-
C. showae	+	+	-		+	V	V	-	V	+	V	-	-
C. sputorum	+	V	-	V	+	+	-	-	+	V	+	V	-
C. upsaliensis	+	-	-	--	+		+	-	V	-	+	-	V

1

Table 1.2 Most common antimicrobials used in nursery age pigs for growth promotion (Reference: USDA National Animal Health Monitoring System, NAHMS), available at http://www.aphis.usda.gov/vs/ceah/cahm/Swine/swine.htm)

Antimicrobials	Percentage of Sites Used	Average No. Days Used
Chlortetracycline	30.1	24.5
Tylosin	23.2	26.7
Carbadox	22.8	23.5
Tiamulin	14.6	16.9
Chlortetracycline/Sulfathiazole/Penicillin Combination	11.5	23.0
Bacitracin	9.1	38.8
Apramycin	8.6	28.2
Tylosin & Sulfamethazine	6.6	23.7

Table 1.3 Percentage of sites gave antimicrobials or other feed additives to grower/finisher pigs, by primary reason and route of administration (Reference: USDA National Animal Health Monitoring System, NAHMS), available at http://www.aphis.usda.gov/vs/ceah/cahm/Swine/swine.htm)

Primary Reason	Route of Administration			
	Feed	Water	Injection	Any Route
Growth Promotion	63.7	0.0	0.0	63.7
Respiratory Disease Treatment	27.4	25.2	57.2	61.9
Disease Prevention	37.9	4.0	6.4	42.8
Enteric Disease Treatment	15.2	7.5	15.4	27.5
Other Disease Treatment	0.2	1.0	14.1	14.7
Any Reason	88.5	31.2	64.5	92.6

Table 1.4 Percentage of sites gave antibiotics by different routes in different site sizes (Reference: USDA National Animal Health Monitoring System, NAHMS), available at http://www.aphis.usda.gov/vs/ceah/cahm/Swine/swine.htm)

Route	Small (<2,000 pigs)	Medium (2,000-9,999 pigs)	Large (>10,000 pigs)
Feed	78.4	87.6	94.1
Injection	38.7	69.8	82.9
Water	18.8	62.5	81.3
Orally	5.9	10.2	7.1

Table 1.5 The five most common antimicrobials given to grower/finisher pigs in feed (Reference: USDA National Animal Health Monitoring System, NAHMS), available at http://www.aphis.usda.gov/vs/ceah/cahm/Swine/swine.htm)

Feed	
Antimicrobials	Percentage of Sites
Tylosin	56.3
Chlortetracycline	48.0
Bacitracin	35.0
Lincomycin	8.6
Carbadox	6.3

Table 1.6 The five most common antimicrobials given to grower/finisher pigs by injection (Reference: USDA National Animal Health Monitoring System, NAHMS), available at http://www.aphis.usda.gov/vs/ceah/cahm/Swine/swine.htm)

Injection	
Antimicrobials	Percentage of Sites
Procaine Penicillin G	40.0
Tylosin	30.7
Ceftiofur	18.2
Ox Tetracycline	18.1
Penicillin	15.5

Table 1.7 The five most common antimicrobials given to grower/finisher pigs in water (Reference: USDA National Animal Health Monitoring System, NAHMS), available at http://www.aphis.usda.gov/vs/ceah/cahm/Swine/swine.htm)

Water	
Antimicrobials	Percentage of Sites
Oxytetracycline	8.8
Chlortetracycline	6.7
Sulfadimethoxine	5.6
Neomycin	4.3
Tylosin	4.1

Table 1.8 Antimicrobial resistance-conferring mechanisms in *C. jejuni* and *C. coli* (Reference: Moore et al., 2005).

Antibiotic	Mechanism of resistance
Aminoglycosides (with the exception of kanamycin)	Chromosomal: enzymatic modification of antibiotics. Integron-mediated resistance
Kanamycin	Majority plasmid-borne, remainder chromosomal; resistance through enzymatic modification of kanamycin
Chloramphenicol	Plasmid-borne, resistance through modification of the target site (ribosome) or alteration of the antibiotic
Ciprofloxacin	Chromosomal: modification of gyrA and parC confers resistance
Erythromycin	Chromosomally mediated, resistance through modification of the target site (ribosome)
β-Lactams	Chromosomal; three mechanisms, decreased uptake through modification of a porin, alteration of a penicillin binding protein, or production of a β-Lactamase
Tetracycline	TetO Gene, plasmid-borne in the majority of cases, resistance mediated through ribosomal protection
Trimethoprim	dfr1 Gene, chromosomal, located to the remnants of an integron dfr9 Gene, chromosomal, located to the remnants of a transposon Resistance arising through modification of the trimethoprim target
Multidrug-resistance (MDR)	Efflux pump with a broad specificity; preventing accumulation of antibiotics

2. Comparison of prevalence and antimicrobial resistance of *Campylobacter* in swine reared in the conventional and antimicrobial-free pig production systems

Abstract

Antimicrobial resistant *Campylobacter* has been reported from conventional swine production systems. However, no study has been done to determine the prevalence of *Campylobacter* in swine reared in the antimicrobial free production system (ABF). It is important to determine and compare the prevalence of this pathogen in production systems and also to determine the association between use of antimicrobials and disinfectants at the farm to the frequency of resistance. To further discern the status of *Campylobacter* in the ABF system, isolates from two different ABF production systems (Intensive and Extensive) were compared at farm and slaughter. *Campylobacter coli* were the predominant species with 1459 (99%) isolates isolated in the study. We found significantly higher pig-level prevalence of *C.coli* at the nursery stage in the ABF (77.3%) than the conventional farms (27.6%) ($P < 0.001$). There was no significant difference in the prevalence between the finishing farms of the two systems. At the slaughter level, we found significantly higher prevalence at post-evisceration than the pre-evisceration stage ($P < 0.001$) in both production systems. All the isolates were tested for their susceptibility to six antimicrobials including chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid and tetracycline using the agar dilution system. Overall, *C. coli* isolates exhibited highest frequency of resistance against tetracycline (Tet, 66.2%) followed by erythromycin (Ery, 53.6%). While frequency of resistance to these two antimicrobials was high in both production systems, it was significantly higher among conventional herds at both farm and slaughter. Resistance against

the fluoroquinolone ciprofloxacin at the highest concentration of (4 mg/L) was also found at the farm level in both the systems with 59% (n = 9) of them having the threonine to isoleucine mutation at amino acid position 86. Eight of the nine *C. coli* isolates, resistant at the highest concentration of initial ciprofloxacin concentration (4 mg/L), also were resistant at the highest concentration of 64 mg/L. Resistance against tetracycline, erythromycin and multiple resistance (resistance to two or more antimicrobials) was found significantly more associated with the conventional production system.

Multidrug resistant (MDR) *C. coli* strains were detected both in the conventional (7%) and ABF (4%) herds. Testing of three *campylobacter* colonies per pig revealed high phenotypic diversity at the level of resistance and in the MIC for the antimicrobials tested. Comparing the two ABF systems, we observed no significant difference in *C. coli* between them at the finishing farm level ($P = 0.83$). At the pre-evisceration stage of the slaughter plant, we observed significantly higher proportion of *C. coli* among the carcass in the extensive reared ABF pigs ($P < 0.001$). At post-chill stage, *C. coli* were isolated only from the extensively reared ABF pigs. Resistance was observed against the fluoroquinolone ciprofloxacin at the farm level in both the production systems. Multidrug resistant (MDR) *C.coli* strains were detected with Ery Nal Tet pattern (3%) being the most common.

This study highlights the high prevalence of antimicrobial resistant *C.coli* in both conventional and ABF systems of pig production. This is the first study reporting the isolation of ciprofloxacin resistant strains from ABF pigs in the United States. The high prevalence of *C. coli* in ABF herds, the common occurrence of antimicrobial resistance in both production systems, particularly resistance to tetracycline and erythromycin coupled with a low frequency but the significant occurrence of ciprofloxacin resistant *C. coli* in both

production types may have significant implications on the antimicrobial use, resistance and persistence of antimicrobial resistant *Campylobacter* in the current pig production and processing environment.

2.1 Introduction

Campylobacter is the leading cause of foodborne bacterial illnesses and is responsible for an estimated 2.4 million illnesses (Mead et al., 1999). The preliminary data as estimated by FoodNet for diseases caused by enteric pathogens for the year 2004 shows *Campylobacter* to have an incidence of 12.9 per 100,000 persons, second only to *Salmonella* with an incidence of 14.7 per 100,000 persons (CDC, 2005). This pathogen is a significant hazard to humans owing to the low infective dose required for infection, high incidence of clinical disease and potentially sequelae in the form of Gullian-Barre syndrome. Although *C. jejuni* in humans is considered to be the most important *Campylobacter* species causing infection, recent studies done in Spain and UK have highlighted the importance of *C. coli* as an important human pathogen due to it ability to show resistance to various classes of antimicrobials and in causing more indigenously acquired foodborne diseases (Saenz et al., 2000; Tam et al., 2003). *C. coli* has been suggested to be particularly suited to the swine environment and has been isolated from pigs on farm in up to 100% of the samples collected (Saenz et al., 2000). Although prevalence on carcasses at the slaughter farm is low compared to the farms, *C. coli* has been isolated at different stages of processing ranging from 2% to 9% at pre-chilling to 1.7% at the post-chilling stage (Korsak et al., 1998; Oosterom et al., 1985; Nesbakken et al., 2003).

Foods of animal origin have been shown to be the major risk factor in causing illnesses in humans (Oosterom et al., 1984). The role of pork products in causing foodborne campylobacteriosis has not been fully elucidated even though *C. coli* have been commonly isolated from pork products in retail markets in the US and Canada (Zhao et al., 2001; Hariharan et al., 1990). Although antimicrobials are not recommended for treating mild campylobacteriosis cases, they are prescribed in complicated systemic cases (Allos, 2001; Picher et al., 1987). The emergence of fluoroquinolone and macrolide resistance in *Campylobacter* species could potentiate the ability of this pathogen to disseminate widely. Resistance to important classes of antimicrobials like the fluoroquinolones used in the treatment of severe cases of campylobacteriosis has reported to be on the rise in the US since 1990 (Gupta et al., 2004; Smith et al., 1999). The report by Gupta et al. also indicated that infection with fluoroquinolone resistant strains of *Campylobacter* prolongs the duration of gastrointestinal infection when compared to infection caused by a susceptible stain (Gupta et al., 2004).

Use of antimicrobials in the feed of pigs and for therapeutic purposes has been shown to be a risk factor for antibiotic resistance in Gram-negative bacteria (Gellin et al., 1989). Dunlop et al. provided evidence that in feed antimicrobials addition was a risk factor for increased resistance in *Escherichia coli* isolated from pigs than individual animal treatment. (Dunlop et al., 1998). The authors concluded that use of antimicrobials in feed does increase resistance to them. In this perspective, the role of antimicrobials used for growth promotion in animals in the development of resistance in pathogens has become a debating issue and is important to determine in *Campylobacter*.

The status of *Campylobacter* in swine raised in the conventional system of production where antimicrobials are used both for treatment and growth promotion has been reported previously in many studies (Looveren et al., 2004; Payot et al., 2004; Saenz et al., 2000). However, there is paucity of information as to the comparative significance of *Campylobacter* occurrence and antimicrobial resistance among pigs reared in ABF and conventional production systems. Under the conventional system of raising pigs, antimicrobials are used both for growth promotion as feed additives as well as for treatment purposes. In the ABF type production system, antimicrobials are used neither for growth promotion nor for treatment and any pig that is treated for an infection by antimicrobials under the ABF system is immediately removed from the group. Studies in the two distinct production systems have been reported for other host species such as poultry and dairy cows in the United States but for swine (Heuer et al., 2001; Sato et al., 2004). The focus of this study was to determine and compare the prevalence and antimicrobial susceptibility of *Campylobacter* in conventional and ABF pig production systems on farm and at slaughter. The second objective of this study was to determine whether use of different antimicrobials and chemical disinfectants at the farm are risk factors for the antimicrobial resistant *Campylobacter*.

2.2 Materials and Methods

2.2.1 Sample collection at farm

Samples were collected on farm from swine reared in two distinct production systems, namely, the conventional and the ABF systems in North Carolina. Farms belonging to these two systems were geographically located far from each other (approximately 50

miles apart). Pigs reared under the ABF system were sampled from two distinct ABF productions systems, namely the extensive and the intensive systems. Farms belonging to these two types of ABF systems were geographically located far from each other and all except one extensive ABF farm were from the eastern part of North Carolina. The biosecurity measures on the extensive ABF farms were not as strict when compared to the intensive farms. This was primarily because the pigs were reared under open sky in a barricaded area, had more human contact and had free access to the environment including soil and water. Under the intensive ABF system, pigs were reared in conventional confinement facilities.

A total of 21 groups of pigs were included in this study and sampled over a period of two years extending from October 2002 to October 2004. At the farm level, fecal samples were collected at nursery (6 to 8 weeks of age) and finishing farms (within 48 hours prior to marketing). Pigs sampled during the study were ear tagged and tattooed for individual identification at subsequent stages of processing at the slaughter plant. Seven group of pigs, including three from conventional and four from the ABF system were sampled at both the nursery and finishing farms. In addition, fecal samples were collected from additional 14 groups (eight conventional and six ABF farms) of pigs at the finishing farms (within 48 hours of marketing). A total of five finishing farms were sampled from each of the two ABF systems making it a total of 10 groups of pigs sampled. At every farm visit, approximately 10 grams of fresh fecal samples was collected per pig with gloved hands directly from the rectum and processed for *Campylobacter* as described later. Fecal samples were transported to the laboratory on ice and processed on the same day upon arrival in the laboratory.

2.2.2 Sample collection at slaughter

Slaughter samples were collected in the form of carcass swabs from two distinct slaughter plants where all the 21 groups of pigs were processed. The first slaughter plant processed both the conventional and ABF pigs and used a blast chiller (-30 ^{o}C / 2 hours) for rapidly cooling the carcasses. The ABF pigs in this plant were processed only on the first day of every week and in the first shift to prevent cross-contamination from conventionally reared pigs. This plant was also cleaned and disinfected every weekend to prevent contamination of carcasses. The second slaughter plant processed only ABF reared pigs and used overnight chilling (1-4 ^{o}C / approximately for 18 hours) for chilling of the carcasses. Sixteen groups (11 conventional and five ABF) were sampled at the first slaughter plant and the remaining five ABF groups at the second plant.

Sample collection at the slaughter plant was done at three different stages; namely, i) pre-evisceration (immediately before evisceration of the gut), ii) post-evisceration (after gut evisceration) and finally, iii) post-chill (after the sample is chilled and ready for packing). Sterile swabs soaked in 10 ml of buffered peptone water (Becton Dickinson, NJ, USA) were swiped along the midline of the carcass extending from the jowl to the ham. One sample swab was collected from each carcass and a total of 10 carcasses per group were swabbed at each of the pre and post-evisceration stages. At the post-chill stage, we sampled 10 carcasses per group with two swab samples from each carcass by using the USDA recommended method (Palumbo et al., 1999) on one side and on the other side by the single swipe method to generate baseline data whether the latter method gives comparative results to the USDA method. Therefore, we collected a total of 40 samples from 30 carcasses from each group of pigs at the slaughter plants, 20 samples from the 10 carcasses each at pre and post-

evisceration stage and another 20 samples from the 10 carcasses at the post-chill stage. Samples were transported to the laboratory on ice and processed on the same day upon arrival.

2.2.3 *Campylobacter* isolation

Fecal sample from the farms were directly plated (loopful) onto campy-cefex selective plates (Pearce et al., 2003) and incubated under microaerobic conditions (CO_2: 10%, O_2: 5% and N_2: 85%) using the Anaeropacks (Remel, KS, USA) at 42^{o}C for 48 hours. All the incubations in subsequent steps were carried out under microaerobic conditions at the same temperature and duration unless stated otherwise. Carcass swabs were first soaked in 30 ml of Bolton broth (Oxoid, Hampshire, UK) and incubated for 48 hours. After incubation, the swabs in each whirl-pack bag were first squeezed and a loopful (approximately 10 µl) of enriched liquid was aseptically withdrawn and streaked onto campy- cefex plates and incubated. Three *Campylobacter* colonies growing on the campy-cefex selective plate from each presumptive positive sample (fecal/carcass sample) were tested biochemically using the catalase (3% H_2O_2 release of oxygen evidenced by bubble formation) and the oxidase tests (tetramethyl-*p*-phenylenediamine; evidenced by color change of colonies) (Becton Dickinson, NJ, USA) for confirmation. Colonies that were positive for both catalase and oxidase tests were streaked onto Mueller-Hinton agar (Remel, KS, USA) plates and further speciated using PCR as explained below. Finally, individual *Campylobacter* isolates with appropriate database numbers were stored at -80^{o}C in brain heart infusion broth (Becton Dickinson, NJ, USA) supplemented with 35% DMSO (Sigma, MO, USA) until further analysis.

2.2.4 *Campylobacter* speciation

We used species-specific primers for PCR detection of important species of *Campylobacter* particularly, *C. coli* and *C. jejuni*. The *ceuE* Gene that encodes a protein involved in siderophore transport was used for detection of *C. coli* while the hippuricase gene (*hipO*) was used for detecting *C.jejuni* (Gonzalez et al., 1997; Hani and Chan, 1995). DNA was purified from freshly grown cultures using the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA). The forward and reverse primers for *ceuE* Gene amplification were CC2- 5'-GATTTTATTATTTGTAGCAGCG-3' and CC3- 5'-TCCATGCCCTAAGACTTAACG-3' (Gonzalez et al., 1997) and for the *hipO* gene amplification were Hip1A-5'-ATGATGGCTTCTTCGGATAG-3' and Hip2B-5'-GCTCCTATGCTTACAACTGC-3', respectively (Hani and Chan, 1995). PCR was done using initial denaturation step at 94 ^{o}C for 5 min followed by 30 cycles of 94 ^{o}C for 1 min, 54 ^{o}C for 1min and 72 ^{o}C for 1 min. A final extension step of 72 ^{o}C for 7 min was done and the reactions were maintained at 4 ^{o}C until separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

2.2.5 Antimicrobial susceptibility testing

The agar dilution method, as recommended by the National Clinical Committee for Laboratory Standards (NCCLS) subcommittee on Veterinary Antimicrobial Susceptibility Testing for determining the resistance /susceptibility of *Campylobacter* strains to different antimicrobials, was used in this study (NCCLS, 1999; Ge et al., 2002). We tested the isolates for their susceptibility against a panel of six antimicrobials. The list of antimicrobial with their abbreviations and range of concentrations used is: Chloramphenicol (Ch; 0.25-128 mg/L), Ciprofloxacin (Cip; 0.008-4 mg/L), Erythromycin (Ery; 0.06-32 mg/L), Gentamicin

(Gen; 0.06-32 mg/L), Nalidixic acid (Nal; 0.25-128 mg/L) and Tetracycline (Tet; 0.06-32 mg/L) (Ge et al., 2003). All the antimicrobials were procured from Sigma (Sigma, MO, USA) except ciprofloxacin (Serologicals Proteins, Inc., IL, USA). The NCCLS breakpoint interpretative criteria for *Enterobacteriaceae* family were used for all the antimicrobials except erythromycin as recommended by NCCLS as the interpretive standard breakpoint levels for the *Campylobacteriaceae* family are not yet available (National Committee for Clinical Laboratory Standards, 1999). For erythromycin (8 mg/L), the breakpoint level used by National Antimicrobial Resistance Monitoring System (NARMS) was adopted (FDA/CDC/USDA, 1999). *C. jejuni* ATCC 33560 was used as the quality control (QC) organism for this test (Ge et al., 2003). The minimum inhibitory concentration $(MIC)_{50}$ breakpoints used for each antimicrobial were: Chloramphenicol (32 mg/L), Ciprofloxacin (4 mg/L), Erythromycin (8 mg/L), Gentamicin (16 mg/L), Nalidixic acid (32 mg/L) and Tetracycline (16 mg/L).

Fresh growth of *C.coli* isolates on sheep blood agar plates was diluted in 3 ml of Mueller-Hinton broth using a colorimeter (bioMerieux, NC, USA) to a concentration of 0.5 McFarland turbidity standards ($1X10^8$ CFU/ml). Two fold serial dilutions of the antimicrobials were made in sterile distilled water with the above mentioned dilution range. One ml of the diluted antimicrobial in 2 ml of sheep blood was added to 17 ml of Mueller-Hinton agar making a total of 20 ml agar media with the desired concentration of the antimicrobial as recommended. The diluted cultures (approximately $1X10^4$ CFU/inoculum) were then plated onto the antimicrobial plates using a Cathra replicator with 1 mm diameter pins (Oxoid Inc., Ontario, Canada). The plates were then incubated at 42^oC for 24 hours and the MIC was recorded for each antimicrobial. Resistance to each antimicrobial was

determined using the recommended breakpoints. Multi-drug resistance (MDR) of isolates here is defined as those isolates that are resistant to three or more antimicrobials. *Campylobacter* isolates showing resistance against ciprofloxacin at the highest concentration of 4 mg/L were further tested at higher concentrations of the antimicrobial up to 64 mg/L.

2.2.6 Molecular characterization of Ciprofloxacin resistant *C. coli* isolates

PCR amplification of the *gyrA* mutation in the quinolone resistance determining region (QRDR) at nucleotide position 86 which codes for the amino acid isoleucine instead of threonine (Th-86 to Ile) was done using the mismatch amplification mutation assay (MAMA) (Zirnstein et al., 2000). Briefly, genomic DNA purification of the isolates for sequencing was done using the Qiagen DNA purification kit (Qiagen, Valencia, CA). The GZ*gyrA*C-coli3F conserved forward primer (5'- TATGAGCGTTATTATCGGTC-3') was used along with the MAMA*gyrA*8 reverse mutation detection primer (5'-TAAGGCATCGTAAACAGCCA-3') to amplify a 192 bp PCR product that was a positive indication for the Thr-86 to Ile mutation. In addition, primers GZ*gyrA*C-coli3F and GZ*gyrA*Ccoli4R (5/- GTCCATCTACAAGCTCGTTA-3') were used to amplify a 505 bp positive control PCR product from the *gyrA* gene. PCR cycling conditions included an initial denaturation of 94^{o}C for 3 minutes, followed by 30 cycles of 94^{o}C for 30 seconds, 54^{o}C for 30 seconds and 74^{o}C for 20 seconds. The final PCR products were run of 1.5% agarose gel mixed with ethidium bromide for analyzing the result.

Based on the MAMA PCR result, all the isolates that did not have the Thr-86 to Ile mutation were further analyzed to look for mutations in the QRDR region. The 505 bp positive control amplicon of the *gyrA* gene was purified from the gel using the QIAquick gel

extraction kit (Qiagen Ltd.). Sequencing reactions using the forward and reverse primers only in separately wells was done using 2 µl of the BigDye Ready Reaction mix (version 3.1; Applied Biosystems, Foster City, Calif.), 0.5 µl of 1:15 diluted primer, 5.5 µl of molecular grade water and 2 µl of the purified PCR product. Sequencing reaction was performed on the automated 3700 ABI capillary sequencer (Applied Biosystems) with running conditions of 30 cycles of 96 ^{o}C for 10 seconds, 50 ^{o}C for 5 seconds and 60 ^{o}C for 4 minutes. Multiple sequence alignments of the test isolates with the ciprofloxacin susceptible QRDR sequence (Accession number: AF092101) was done using the ClustalW program (www.ebi.ac.uk/clustalw).

2.2.7 Questionnaire survey

Questionnaires forms were handed to the producers at the time of sample collection at the farm. Information was collected from the producers regarding the use of antimicrobials at the farm either for therapeutic or for growth promotion purposes. A total of 13 forms were collected and returned including nine from the conventional system and four from the ABF system. Information collected on the use of antimicrobials included the type of antimicrobials, dose and the route of application. Additional data on the use of disinfectant at the farm during the time when the barns were unoccupied in between pig flow.

2.2.8 Statistical Analysis

Campylobacter prevalence, frequency of antimicrobial resistance profiles and patterns between and within the conventional and the ABF production systems at the farm and slaughter level were compared using the χ^2 test (Minitab Inc. PA, USA) and Fisher's

exact two-tailed test (Langsrud, 2004) wherever applicable. Significant association of antimicrobial resistance profile and patterns with the production system and the processing stage (farm or slaughter) was done using the odds ratio test. A value of $P < 0.05$ was considered statistically significant.

Multiple logistic regression analysis was done to determine the risk factors for antimicrobial resistance in *Campylobacter* against erythromycin, tetracycline and multiple drug resistance (resistance to two or more antimicrobials simultaneously). We decided to test for the above resistance profiles since frequency of resistance against the tetracycline and erythromycin was very high in *C. coli* isolated from the farms. Four antimicrobials that were used either as injectibles (Penicillin and Naxel) or in feed (Oxytetracycline and Tylan) were included in the analysis. Information for the disinfectant use was also included in the analysis. Univariate analysis for testing whether the covariates of interest were significantly different for the two production systems for the outcome of interest (antimicrobial resistance) in order to screen their association and to determine if they should be included in further modeling. We used the chi-square test for this purpose and any explanatory variable having a chi-square *P* value less than 0.25 was included in further modeling.

We considered two modeling approaches for the purpose of determining risk factors for antimicrobial resistance in *Campylobacter*. The first was modeling using the Hierarchical well formulated (HWF) modeling strategy using backward elimination process. The other was generalized estimating equation (GEE) approach for correlated data. This modeling approach was considered with the assumption that resistance profiles among isolates from the same pig were correlated. However, based on the phenotypic heterogeneity (MIC results for the different antimicrobials) as well as genotypic diversity (PFGE and MLST results), we

decided to treat each isolate of *C. coli* as an independent unit. Therefore, for the final modeling we used the HWF modeling approach.

Using the backward elimination process, the interaction terms were first tested and kept in the model if found significant. Interaction terms were tested using the likelihood ratio test. This was done by comparing the likelihood ratio statistics of the full model, including the interaction term, with that of the reduced model (i.e., without the interaction term). If the likelihood ratio test was significant ($P < 0.05$), the interaction term was left in the model. If any interaction term was found significant, then the lower order components of the interaction term were kept in the model. Testing for potential confounders was done using the gold standard model where all the variables including the confounding variable that is to be tested. All further models were compared to the gold standard model as this model controls for all potential confounders. To evaluate whether a potential confounder should be removed, we considered whether the odds ratio for the gold standard model was meaningfully different (change of 20% or more in the odds ratio value of the main exposure variable) from the odds ratio of the reduced model, which included all variables except the potential confounder. Any variable that caused a meaningful change in the odds ratio was evaluated for its effect on the model's precision. Variables that positively impacted the precision of the model were kept as confounders. In case we did not find any significant interaction term, the same strategy was adopted to test for confounders using the gold standard estimate.

Three different models with outcome being tetracycline resistance (Model A), erythromycin resistance (Model B) and resistance to two or more antimicrobials simultaneously (Model C) were developed. Three *C. coli* isolates were isolated per pig

positive for *Campylobacter*. The outcome for all the three models was at the isolate level with isolates being selected representing the finishing farms. Although collecting three *Campylobacter* isolates from each positive pig was attempted, there were *Campylobacter* positive pigs we could recover less than three colonies. Resistance testing was done as described in section 2.2.5. The list of antimicrobial used for growth promotion and therapeutic use at the farm level for the conventional production system is shown in Table 2.12. The table also includes use of chemical disinfectant at these farms. However, use of antimicrobials on pigs at farm level was restricted to the conventional production system only. Since no antimicrobials were used in the ABF system, we decided to use conventional production system as a proxy for antimicrobial use at the farm and was coded as "1". The ABF system was coded as "0". Similarly, the use of antimicrobial or chemical disinfectant at the farm was coded as "1" while absence of use was coded as "0". For the final model analysis, the only two variables that were offered in the model were the main exposure variable (production system) and use of chemical disinfectant at the farm. All analyses were done using the Egret software (version 2.0.3, Cytel Corp., Cambridge, MA) and Minitab (Minitab Inc. PA, USA).

2.3 Results

2.3.1 *Campylobacter coli* prevalence

2.3.1.1 *C. coli* prevalence comparison between the two systems

This cross-sectional study was conducted to determine the prevalence and antimicrobial resistance profile of *Campylobacter* species present at farm and slaughter in swine raised in the conventional and the ABF production systems. For this purpose, a total of

908 pigs and 562 carcasses originating from 21 pig groups were sampled at farms and slaughter plants in North Carolina. *Campylobacter* was isolated from all the farms and slaughter visits. Out of the 1634 *Campylobacter* isolates recovered from this study, 1472 (1117 on farm and 355 from slaughter) isolates were speciated by species-specific PCR approach. The remaining 162 *Campylobacter* isolates could not be regrown again despite multiple attempts. *C. coli* accounted for 99% (1459) of these isolates. None of the remaining 13 isolates were *C. jejuni* and were not included in the subsequent characterization.

On comparing the two production systems at the nursery farm, we found significantly higher pig-level prevalence of *C. coli* in the ABF (77.3%) than the conventional farms (27.6%) ($P < 0.001$) as shown in Figure 2.1. This difference was mainly attributed to the difference in prevalence from the nursery farms and processing (slaughter) stages as no significant difference in the prevalence of this pathogen between the two systems was detected at the finishing farm level (53% and 55.8% for the ABF and conventional respectively). We observed contrasting results when comparing the prevalence within individual production systems at the farm level. Within the conventional system, there was a significant increase ($P < 0.001$) in the prevalence when the pigs were sampled at the finishing (53%) than at the nursery level (27.6%). On the other hand in the ABF system of production, significantly higher ($P < 0.001$) proportion of pigs at the nursery (77.3%) had *Campylobacter* than the finishing level (55.8%).

At the slaughter stage, there was a significantly higher recovery of *Campylobacter* at post-evisceration than the pre-evisceration step. Chilling significantly reduced the recovery of *Campylobacter* in all groups ($P < 0.002$). The USDA and the single swipe carcass swabbing methods, resulted in a similar level of *Campylobacter* recovery in the post-chill

samples (n=195) with 3.6% and 2.6% of the swabs were positive for *Campylobacter* in the USDA and single swipe method respectively. *C. coli* was recovered on carcasses from the two slaughter plants with higher isolation observed from carcasses that undergone the overnight cooling step compared to the 2-hour blast chilling followed by an over-night chilling system ($P < 0.001$).

2.3.1.2 *C. coli* prevalence comparison within the two ABF systems

The overall *Campylobacter* prevalence at the farm and slaughter level was 55.8 % and 26 % respectively. Comparison was made at the finishing farm and slaughter level. All the 532 *Campylobacter* isolates in this study including 366 from farm and 166 from slaughter were *C. coli.* There was no significant difference in *C. coli* prevalence at the farm level for the extensive (55 %) and intensive (56.3 %) rearing systems as shown in (Figure 2.2, $P = 0.83$). However, we did observe significantly higher proportion of *C. coli* at the pre-evisceration stage of processing extensive reared ABF pigs ($P < 0.001$). In both the ABF systems, there was increase in *C. coli* prevalence at post-evisceration followed by a significant decrease at the post-chill stage ($P < 0.002$). Within individual system, we did not observe any difference in prevalence at the post-chill stage between the USDA method and the single swipe method of carcass swabbing. However, on comparing the two ABF systems at the post-chill stage, we observed *C. coli* only from the carcasses of extensively reared ABF pigs.

2.3.2 Antimicrobial resistance profile of *C. coli* isolates

2.3.2.1 Antimicrobial resistance profile comparison between the two systems

We compared the distribution of antimicrobial resistant *C. coli* isolates between and within the two production systems. We detected resistance to all the six antimicrobials tested. Irrespective of the production system and production stage, *C. coli* isolates exhibited highest frequency of resistance against tetracycline (66.2 %) followed by erythromycin (53.6 %) as shown in Table 2.1. We detected significantly higher proportion of isolates from the conventional than the ABF system showing resistance to the above two antimicrobials at the farm level (nursery and finishing both) and also at the pre and the post-evisceration stages of the slaughter plant ($P < 0.005$). Resistance profile of *C. coli* isolated from the processing stages within the production systems is shown in Table 2.2.

In the ABF system, significantly higher frequency of resistance to tetracycline was observed in isolates from the farms (56.3 %) than from the slaughter plants (45.1 %) ($P < 0.001$). Also, *C. coli* isolates from the ABF system showed significantly higher resistance against tetracycline at the nursery level while the opposite was observed in their resistance against erythromycin at the farm level ($P < 0.001$). Higher proportion of isolates from the post-evisceration step were resistant to the above two antimicrobials than at the pre-evisceration step ($P < 0.05$). On comparing the two production systems at farm and slaughter, tetracycline and erythromycin resistant *C. coli* isolates were 3.6 and 6.7 times more associated with the conventional system than the ABF (95% CI: 1.41-1.61 for erythromycin; and 5.36-8.61 for tetracycline; $P < 0.001$; Table 2.3) further signifying the association of antimicrobial use at farm to resistance.

Resistance against ciprofloxacin was detected in *C. coli* isolates from on-farm specimens from both the conventional (2.9 %) and ABF (0.6 %) herds (total n=17). Ciprofloxacin resistant *C. coli* was 4.3 times more associated with the conventional

production system (95% CI: 1.47-12.6) (Table 2.3). Similar observations were made at the farm level with ciprofloxacin resistant isolates 4.8 times (95% CI: 1.6-14.2) likely to be associated with conventional system than the ABF farms. However, we found no difference at the slaughter level. All the ciprofloxacin resistant isolates were also resistant to nalidixic acid. Ten out of 17 isolates were resistant at the highest concentration of 4 mg/L of ciprofloxacin tested. Significantly high frequency of resistance was observed in the conventional production system ($P < 0.05$) at farm and slaughter. However, there was no significant association between nalidixic acid resistance and the conventional system. Within the two systems, resistance to this antimicrobial was significantly more in the nursery than finishing stage under the conventional system ($P < 0.001$). Resistance against gentamicin and chloramphenicol was less frequent and observed in 0.4 and 1.8% of the total isolates (n = 1459) respectively.

2.3.2.2 Antimicrobial resistance profile comparison within the ABF system

Overall, isolates exhibited highest frequency of resistance against tetracycline (48.3 %) and erythromycin (39.2 %) (Table 2.4). On comparing the resistance profile of isolates from the extensive and intensive ABF systems, we detected significantly higher proportion of *C. coli* isolates from the intensive system resistant to the above two antimicrobials at the finishing farms ($P < 0.001$). Except tetracycline resistance among the extensive ABF carcasses, higher proportions of *C.coli* isolates from the ABF system at the post-evisceration step were resistant to tetracycline and erythromycin than at the pre-evisceration step ($P < 0.05$). Resistance against ciprofloxacin (0.5 %) was detected in isolates from on-farm specimens and in both types of ABF herds. As expected, these isolates were also resistant to

nalidixic acid. Gentamicin and chloramphenicol resistant isolates were rare and observed in 0.1 and 1.8 % of the total isolates.

2.3.3 Antimicrobial resistance patterns of *Campylobacter* isolates

2.3.3.1 Antimicrobial resistance pattern comparison between the two systems

We observed 19 different resistance patterns exhibited by 1152 (78.9%) of the isolates as shown in Table 2.5. Significantly higher proportion of isolates from the ABF system (32.8 %) at the farm and slaughter were pansusceptible than those from the conventional system and in conjunction with the univariate analysis shows a protective effect of the ABF system on pansusceptible phenotype (5.2 %) (OR 0.11, 95% CI: 0.07-0.16, Table 2.6). Ery Tet was the most common resistance pattern. This pattern was common regardless of the type of production system. However, higher proportion of isolates from finishing farms (60.6 % and 21% in conventional and ABF respectively) exhibited the Ery Tet pattern than the nursery farms (47 % and 15 % in conventional and ABF respectively). This pattern was 6 times more associated with the conventional production system (95% CI: 4.7-7.6). At the processing stage, Ery Tet was 8.1 and 5.1 times more associated at farm and slaughter for isolates from the conventional system respectively. Frequency of resistance patterns from farm and slaughter stages are shown in Table 2.7. Within both the production systems, significantly higher percentage of isolates had the Ery Tet resistance pattern at the finishing than the nursery level ($P < 0.05$). At the slaughter level, significantly more isolates from the ABF system were found resistant to Ery Tet at the post-evisceration than the pre-evisceration stage ($P = 0.01$). However, this was not so in case of the isolates from the slaughter plant under the conventional system ($P = 0.13$). We observed 11 different MDR patterns with

resistance to three or more antimicrobials in 79 (5.4 %) of the isolates with Ery Nal Tet (2.7 %) being the predominant one. *C. coli* isolates from the conventional system, both on farm and from slaughter, were more often MDR than isolates from the ABF system ($P = 0.005$). Other MDR patterns including Ch Ery Gen (n=1), Cip Gen Nal Tet (n=1), Ch Ery Nal Tet (n=1) and Ch Ery Nal (n=1) were isolated only at the slaughter stages. The frequency of different MDR patterns observed is shown in Figure 2.3.

2.3.3.2 Antimicrobial resistance pattern comparison within the ABF system

Fourteen different resistance patterns were observed with resistance being exhibited to either a single antimicrobial or a combination of them (Table 2.8). A total of 188 isolates (35.3 %) were pansusceptible. On comparing the two ABF systems, significantly higher proportion of isolates from extensive system were found to be pansusceptible than those from the intensive farms ($P < 0.001$). The most common resistance pattern observed in this study was resistance to erythromycin and tetracycline, Ery Tet. This pattern was significantly higher exhibited in *C. coli* isolates from the intensive system both at farm and slaughter ($P < 0.001$). We observed six MDR patterns in 24 (4.5%) of the isolates with Ery Nal Tet (3 %) being the predominant one. Except for one isolate with Ch Ery Nal Tet and another with Ch Ery Gen resistance pattern isolated at the post-evisceration step, none of the isolates at the slaughter level were MDR.

2.3.4 Comparison of MIC Levels of antimicrobials

2.3.4.1 MIC Levels comparison across the two production systems

MIC levels were further analyzed to determine if there is a variation in MIC amongst resistant isolates assessed based on breakpoints as described above (Table 2.9). Such comparison, however, may not be conclusive as the strains may exhibit a one dilution difference in MIC though they are clonal. One antimicrobial that was of interest in the MIC analysis was chloramphenicol. Even though comparable frequency of resistance to chloramphenicol among *C. coli* observed in both on-farm and at slaughter stages, isolates from the slaughter plants were resistant to chloramphenicol at a four fold higher MIC (128 mg/L) than from the farms implying that a different chloramphenicol resistant strains may be recovered at different stages of the production to processing continuum. No variation is MIC has been detected against tetracycline, the antimicrobial to which resistance was the most common. Except for isolates from the ABF slaughter plant that were mostly susceptible at MIC of 4 mg/L (21 %), resistant isolates from both the production types exhibited an MIC of 32 mg/L of tetracycline at all stages of production. However, variation in MIC for the second common one, erythromycin was found.

Most of the isolates (farm 68.9 % and slaughter 69.8 %) from conventional system were resistant against erythromycin even at the highest concentration of 32 mg/L. Isolates from the ABF system (28.3 % farm and 27.6 % slaughter) were mostly grouped in the MIC of 2 mg/L, exhibiting a 16 fold reduced MIC when compared to isolates from the conventional system. In regards to ciprofloxacin resistance, 10 out of the 17 resistant isolates exhibited resistance to the highest concentration of ciprofloxacin tested (4 mg/L) tested in this study. Higher proportions of isolates from the ABF farms (3.3 %) were resistant to nalidixic acid at the maximum concentration of 128 mg/L than those from the conventional farms (1.1 %).

2.3.4.2. MIC Levels comparison within the ABF production system

For chloramphenicol, MIC observed was higher on farm than at slaughter. Higher proportion of *C. coli* isolates from the extensive production system had an MIC of 4 mg/L both at farm and slaughter (Table 2.10). Within the extensive production system, higher proportion of isolates from the slaughter plant had this MIC than those from the farms thus implying that different chloramphenicol resistant strains may be recovered at different stages of the production to processing continuum. Significant difference was observed when the MIC for isolates resistant to erythromycin and tetracycline were compared across the two systems. Significantly higher proportion of *C. coli* isolates from the intensive system was resistant at an MIC of > 32 mg/ L concentration for the two antimicrobials both at farm and slaughter ($P < 0.001$). Higher proportions of isolates from the intensive ABF farms (8.8 %) were resistant to nalidixic acid at the maximum concentration of 128 mg/L than those from the intensive farms (1.2 %). Ciprofloxacin concentration of 0.125 mg/L was the MIC where highest proportions of isolates from both the ABF systems were clustered except for the slaughter isolates from the intensive system (MIC 0.06 mg/L).

2.3.5 Phenotypic diversity (Heterogeneity) among isolates from the same pig/ carcass

2.3.5.1 Heterogeneity based on MIC Levels

As mentioned previously, three isolates per positive samples were selected randomly and further tested for their resistance to six antimicrobials. Heterogeneity at the phenotypic level taking two-fold and four-fold differences in the MIC was observed at the farm and slaughter levels of both the production systems. Overall heterogeneity based on the MIC

level was observed in 39 % and 60.5% at four and two-fold difference, respectively, of the samples as shown in Table 2.11. Higher proportion of isolates from an individual pig or carcass from the ABF herd showed heterogeneity based on the two and four-fold MIC levels than the conventional herds ($P < 0.05$) except at the slaughter for the four-fold difference ($P = 0.83$). On comparing isolates from the farm and slaughter plants in the both the production systems, we observed no significant difference between the MIC levels at two and four-fold difference ($P < 0.6$).

2.3.5.2 Heterogeneity based on the resistance patterns

Based on the resistance patterns, phenotypic diversity between the two systems was not significantly different ($P = 0.45$). Overall heterogeneity based on the resistance patterns was observed in 32% of the samples as shown in Table 2.11. There was no significant difference between heterogeneity seen in resistance patterns within each production system at farm and slaughter (Conventional system, $P = 0.21$; ABF system, $P = 0.09$).

2.3.6 Molecular characterization of Ciprofloxacin resistant *C. coli* strains

Ten out the 17 ciprofloxacin resistant *C. coli* isolates were positive for MAMA PCR indicating the point mutation at amino acid position 86 with threonine (Thr) replaced by isoleucine (Ile) (Figure 2.4, Table 2.15). Nine out of these 10 isolates were resistant at the highest concentration of > 4 mg/L indicating high resistance. All the MAMA PCR positive isolates were isolated from the nursery stage under the conventional production system. The remaining resistant isolates had an MIC of 4 mg/L. Sequencing of QRDR region of the *gyrA* region for all the 17 isolates were done to further determine the presence of any other

mutation besides Thr-86 –Ile. Multiple sequences comparison of the QRDR sequence region of the isolates with reference *C. coli* (Accession number: AF092101) revealed a point mutation in isolate ID 554 at amino acid position 147 with glutamic acid replaced by aspartic acid (GAA replaced by GAC) and two silent mutations in isolates 554 and 556 at positions 116 (alanine; GCA replaced by GCT) and 108 (glycine; GGA replaced by GGC) respectively (Figure 2.5; Table 2.15). The remaining isolates, including the MAMA PCR negative isolates, did not reveal any mutation in their QRDR region sequences.

Ciprofloxacin resistant *C. coli* isolates that were resistant at the highest concentration of 4 mg/L were further tested by the same method up to 64 mg/L concentration to determine their MIC. Five out of the 10 *C. coli* isolates were again found resistant at the highest concentration tested while two isolates had an MIC of 64 mg/L (Table 2.15).

2.3.7 Risk factor analysis for antimicrobial resistance in *Campylobacter*

Information about the use of antimicrobials for growth promotion and therapeutic purposes and disinfectants for cleaning the farms was available for a total of 13 farms including nine farms of conventional type and four from the intensive ABF system. None of the ABF farms used antimicrobials for growth promotion or therapeutic purposes. However, the intensive ABF farms did use chemical disinfectant on the floor between flows of pigs. A total of 466 *C. coli* isolates (285 from conventional and 181 from the ABF finishing farms) representing the farms for which information of antimicrobial and disinfectant use was available were identified for the analysis. Univariate analysis results used as screening for variables to be included in the model are shown in Table 2.13.

The conventional production system was found to be significantly associated with all the three outcomes of interest (Table 2.14). For model A (erythromycin resistance), conventional production system was 2.3 times more likely to be associated than the ABF production system (OR: 2.3; 95% CI: 1.5-3.6), 5.1 times associated with model B (tetracycline resistance) (OR: 5.1, 95% CI: 3.2-8.1) and 2.1 times associated with model C (resistance to two or more antimicrobials) (OR: 2.1, 95% CI: 1.4-3.1). There was also no evidence that chemical disinfectant use was a confounder.

2.4 Discussion

Thermophillic *Campylobacter* including *C. jejuni* and *C. coli* have been shown to be important bacterial foodborne pathogens and are widely prevalent in animals (Mead et al., 1999; Padungton and Kaneene, 2003). *Campylobacter* has been reported from pigs on farm and from pig carcasses in slaughter plants in many studies done previously (Payot et al., 2004; Looveren et al., 2004; Harvey et al., 1999; Hariharan et al., 1990; Nesbakken et al., 2003; Chang et al., 2003; Pearce et al., 2003). However, these studies have been restricted to pigs reared under the conventional system where antimicrobials are routinely used for treatment and growth promotion purposes. It is important to conduct a comparative analysis of prevalence and resistance between conventional and ABF production systems to better understand the association of antimicrobial use and resistance in the swine production and processing environment.

In previous studies, swine has repeatedly been implicated as important carriers of *C. coli*. Higher prevalence of *C. coli* has been reported from pigs in conventional system ranging from 57.8% in newborn piglets to 100% in pigs (Young et al., 2000; Saenz et al.,

2000). Consistent with previous reports, *C. coli* was the predominant *Campylobacter* species isolated from 99% of the total isolates cultured in this study. We found high prevalence of *C. coli* in conventional as well as ABF herds. This finding emphasizes the importance of pigs as reservoirs of this species regardless of antimicrobial use status in the production environment. Previous studies conducted in the broiler industry have reported a significant difference between the two production systems with higher prevalence of this pathogen in the ABF system (Avrain et al., 2003; Heuer et al., 2001). Heuer et al., reported a prevalence of 91% and 4.5% in the outdoor reared organic poultry flock and 86.2% and 10.3% for the conventional flock for *C. jejuni* and *C. coli*, respectively, and reported a significant difference between the two production systems with higher prevalence of this pathogen in the ABF system (Heuer et al., 2001). A study done in dairy cattle reported no significant difference in the prevalence of *C.coli* between the organic and the diary herds (Sato et al., 2004).

Increase in the prevalence with age in conventional pigs, in the current study, could be attributed to the differences in the rearing conditions and associated risk factors, an area beyond the scope of this study. Decrease in the carriage of *Campylobacter* with age, as seen in the ABF system in this study, has been reported previously in pigs and dogs before (Weijtens et al., 1997; Young et al., 2000; Matsusaki et al., 1986). Within the ABF system, high prevalence in extensively reared nursery pigs could be attributed to horizontal transmission via the open environment where the pigs have unrestricted access to the soil and water. *C. coli* have shown to be present in the environment in both soil and water (Leatherbarrow et al., 2004). Horizontal transmission of *Campylobacter* from environmental sources has been suggested to be an important route for the spread of infection in food animals especially poultry (Heuer et al., 2001; Jacob-Rietsma, 1995).

At the slaughter plant level, we observed significant increase in the recovery at the post-evisceration stage followed by a significant decrease in the post-chill stage. The significant increase in recovery of *Campylobacter* from post-evisceration swabs may implicate the significance of various manipulations during and after evisceration including potential gut spillage, cross-contamination and other external sources. Higher recovery of *C. coli* at the post -evisceration than the pre-evisceration step of the slaughter plant was observed and could be because the post-evisceration stage involves taking out the gut from the inside which can contaminate the carcass. The gastrointestinal tract of the pig is an important reservoir of *Campylobacter* with prevalence of 100% in the gastrointestinal tract (Nesbakken et al., 2002) and concentration in $\log_{10}$ colony forming unit (cfu) per gram feces varying from 5.08 in gilts, 7.25 in weaned piglets to 5.31 in pregnant sows (Young et al., 2000). The mean log number of 3.6 cfu in adult pigs has been reported from many studies (Young et al., 2000; Oosterom et al., 1985; Weijtens et al., 1993). Studies done previously in slaughter plants have reported *Campylobacter* prevalence ranging from 2-9% on pig carcasses after evisceration (Korsak et al., 1998; Pearce et al., 2003; Oosterom et al., 1985). Significant reduction seen at the post-chill stage in both the production systems was expected since *Campylobacter* is highly susceptible to cold and drying conditions as reported previously (Oosterom et al., 1985; Chang et al., 2003).

Between the two ABF type systems, *Campylobacter* isolates could be isolated at the post-chill stage only from the carcasses belonging to the extensively reared system using both the Chill-USDA and the single swipe method of sample collection. This could be due to the slaughter house effect with the extensively reared ABF pigs being slaughtered in the plant that employs the 24-hour freezing method. The slaughter house where the intensively reared

pigs were slaughtered used a blast-chiller system where the carcass is chilled at -35^{o}C temperature for two hours before it is kept in a regular chiller unit. This blast chiller uses a rapid cooling of the carcasses within 2 hours that may have more deleterious effect on survival of pathogens that the 24 hour cooling method. Comparison of two carcass swabbing methods, USDA and single swipe showed no difference in the *C. coli* isolation from carcasses at the post-chill stage. We recommend conducting a more thorough study focusing on different foodborne pathogens and with a larger sample size to obtain more confirmatory results. If the two methods are found to have no difference in their ability to recover *Campylobacter* and other important pathogens, as described in this preliminary analysis, then a less cost, time and labor-intensive method of single swipe along the midline can be recommended for future use.

C. coli could be isolated at the post-chill stage by using both the Chill-USDA and the single swipe method only from the carcasses belonging to the ABF pigs slaughtered on the second slaughter plant. This could be due to the slaughter house effect since the plant employed the 24-hour chilling method in contrast to the blast chilling method followed by over night chilling employed by the first plant that slaughtered all the conventional herds. Previously, an experimental study conducted by Chang et al., reported blast chilling method to be more effective than the conventional chilling method to significantly reduce *C. coli*, *Salmonella* Typhimurium and *Listeria monocytogenes* on pork carcasses (Chang et al., 2003).

Antimicrobial resistance was most commonly found against tetracycline and erythromycin similar to reported by other studies (Delsol et al., 2004; Pezzotti et al., 2003; Van Looveren et al., 2004; Payot et al., 2004). There was a significant difference in

resistance to these antimicrobials between the two production systems. Higher frequency of resistance was detected in conventional herds than the ABF consistent with the antimicrobial use status indicating the association between antimicrobial use and resistance. Chlortetracycline and the macrolide tylosin are the two most commonly used antimicrobials in the conventional swine production system for growth promotion purposes (National Animal Health Monitoring System, 2002). In the conventional farms sampled, oxytetracycline and tylan were used in feed for growth promotion purposes at the nursery and finishing stages. The absence of antimicrobial selective pressure in the ABF system could be the reason for the lower proportion of resistant *C. coli* isolates. Though the frequency of resistance for these two antimicrobials was relatively lower in ABF herds, high proportions of the isolates from the ABF herds were resistant to both tetracycline and erythromycin (56.2% and 36.6% for Tet and 34.5% and 40.4% for Ery on-farm and at slaughter respectively). Significantly higher proportion of tetracycline resistance seen on farm compared to slaughter points to different sources, particularly the environmental sources, which may be transmitting these resistant strains at the farm level. Previous studies have reported the temporal relationship between use of antimicrobials and the emergence of antimicrobial resistant strains of pathogens (Engberg et al., 2001; Gupta et al., 2004; Padungton and Kaneene, 2004). Similar study comparing the two production systems in broilers also reported significantly higher resistance against tetracycline and erythromycin in the conventional than the organic production system (Heuer et al., 2001; Desmonts et al., 2004; Avrain et al., 2003).

This is an important finding that may have some implications including the fact that in the absence of antimicrobial selective pressure, resistant strains could prevail. It may be

attributed to in-plant cross contamination of *Campylobacter* between different pig herds. This is especially likely since none of the slaughter houses where the samples were collected were dedicated for ABF herds. Thus, the likelihood of cross contamination at lairage and processing could be high. Some of the reasons for persistence of resistant strains in ABF herds could be co-selection of antimicrobial resistance with other phenotypes not directly related to antimicrobial use or the potential for resistant strains to be similarly fit to survive in the production environment as that of the susceptible ones thus resulting in lingering of resistant strains for an extended period of time. Previous studies have reported the temporal relationship between use of antimicrobial use and the emergence of antimicrobial resistant strains of pathogens (Gupta et al., 2004; Padungton and Kaneene, 2003; Engberg et al., 2001). Similar study comparing the two-production system in broilers also reported significantly higher resistance against tetracycline and erythromycin in the conventional than the organic production system (Desmonts et al., 2004; Heuer et al., 2001; Avrain et al., 2003).

Resistance against erythromycin is concerning since it is the drug of choice besides ciprofloxacin for treating severe cases of campylobacteriosis in humans (Sanchez et al., 1994). Comparison of the MIC levels for both the above antimicrobials in the two systems revealed an interesting picture. Maximum number of isolates isolated from the ABF system had a 16 fold lower MIC than their counterparts from the conventional system. Desmonts et al., reported similar results in broilers where majority of the *C.coli* isolates from the free range broilers (antimicrobial free) had lower MIC for erythromycin than those from the conventionally reared broilers (Desmonts et al., 2004).

Resistance against the fluoroquinolone ciprofloxacin was also detected at the farm level in both the conventional and the ABF production systems. This is a very important and interesting finding keeping in mind that ciprofloxacin resistant *C.coli* has not been reported from ABF pigs previously in the United States. Also, no fluoroquinolone antimicrobials were used in either of the two production systems. Therefore, detection of these strains may indicate the possible role of environmental cross contamination, via other risk factors such as exposure to other reservoir animals. Ciprofloxacin resistant strains have been reported in 14%, 17% and 100% of the *C.coli* strains in previous studies done in the non-U.S. studies (Bywater et al., 2004; Van Looveren et al., 2004; Saenz et al., 2000). A recent study conducted in poultry reported that FQ resistance in *Campylobacter* can persist even in the absence of FQ selective pressure (Price et al., 2005).

Ciprofloxacin resistant isolates (n = 17) in this study had an MIC of 4 (n = 7) or were resistant at the highest concentration of 4 mg/L (n= 10) tested during the study. These isolates were also resistant to Nalidixic acid. Ten isolates were positive for Thr-Ile mutation as detected by the MAMA PCR. Five out of the seven isolates positive for the MAMA PCR when tested at higher concentrations of ciprofloxacin were again found to be resistant at 64 mg/L. This implies that the Thr-86-Ile mutation confers high resistance to the isolates. The bimodal nature of resistance seen here has been reported before by Delsol et al. (Delsol et al., 2004). Mutation at amino acid position 86 has been shown to confer high resistance to this antimicrobial at concentrations ranging from 32 mg/L up to 128 mg/L (Griggs et al., 2005; Piddock et al., 2003). A single isolate carried another mutation at position 147 in addition to mutation at position 86.

We do not know at this stage whether the additional mutation has potentiated the ability of this isolate to resist even higher concentration of ciprofloxacin compared to isolates that carry mutation only at position 86. Other mutations in the QRDR that have been linked to ciprofloxacin resistance in *Campylobacter* include Asp-90, Ala-70 and Pro-104 mutations (Engberg et al., 2001; Piddock et al., 2003). Recently, high resistance to moxifloxacin, a fluoroquinolone, was shown in *Campylobacter* isolates that had a double mutation in the QRDR of the *gyrA* gene. Isolates with a single mutation in this region were susceptible (Ruiz et al., 2005). Zhang et al., have also shown the role of CmeABC efflux pump to be involved in maintaining high level of fluoroquinolone resistance (Zhang et al., 2003). Silent mutations have been reported previously in Finland and United Kingdom but have no apparent role in causing resistance against fluoroquinolones (Hakanen et al., 2002; Piddock et al., 2003).

Ery Tet being the most common resistance pattern was not surprising since the isolates were resistant to these two antimicrobials more than any other in this study. Ery Nal Tet was the most common MDR pattern (with resistance to three or more antimicrobials) and has been reported previously by Payot et al., to be the most common MDR pattern in *C. coli* isolates (Payot et al., 2004). Randall et al., reported 3.8% of *C. coli* and 3.3% of *C. jejuni* isolates of human and animal origin to be MDR (Randall et al., 2003). *C. coli* have been found to be more MDR in nature than *C. jejuni* ranging from 17% to 37% (Van Looveren et al., 2001; Payot et al., 2004). MDR resistance patterns in our study were mostly restricted to isolates from the farm. However, a few MDR patterns were observed at the slaughter level and included Cip Gen Nal Tet, Ch Ery Nal Tet, Ch Ery Gen and Ch Ery Nal were observed at the pre and post-evisceration stages. There were no MDR strains isolates from the post-chill step.

Relatively lower number of isolates showing resistance to chloramphenicol and gentamicin is in agreement with other studies that have reported similar low levels of resistance against both the above antimicrobials (0%) in *C.coli* isolates from pork (Ge et al., 2003) and against gentamicin (0 and 3.3%) in pigs (Bywater et al., 2004; Van Looveren et al., 2004). Resistance observed against chloramphenicol was again noteworthy since this antimicrobial has not been used in the U.S. for the last two decades. This highlights the possible role played by environmental factors like soil and water in the spread of these resistant strains.

We tested three colonies per positive sample for their antimicrobial resistance profile and the MIC levels for the six antimicrobials tested. Heterogeneity among *C.coli* isolates was observed at both MIC difference and resistance pattern level. Previous study done by Payot et al. reported different MIC profiles in 76% of the pigs tested (Payot et al., 2004). This diversity existing between isolates from the same animal has been shown to exist at the genotypic level using pulsed field gel electrophoresis by Hume et al. and Enterobacterial Repetitive Intragenic Consensus-PCR by Weijtens et al. (Hume et al., 2002; Weijtens et al., 1999). Our study further highlights the important of testing more than one colony per positive sample for antimicrobial resistance testing so as not to miss any epidemiological link.

Significant association of different antimicrobials including erythromycin, tetracycline and resistance to two or more antimicrobials was found for the conventional production system after univariate analysis. Use of antimicrobials at the farm has been shown to select for antimicrobial resistance (Dunlop et al., 1998). It is important to determine other risk factors that besides the use of antimicrobials at the farm may provide selective pressure

for resistance development in the pathogens. A recent study conducted in pig farms has shown that lameness, shortened tails, ad libitum feeding and use of partial all-in and all-out system of pig flow are associated with resistance in *C. coli* (Schuppers et al., 2005). However, in this study we could only model antimicrobials and disinfectant use at the farm level were associated with resistance.

For all the three models, we found resistance profile and resistance patterns exhibited by *C. coli* isolates to be likely associated with the conventional system than the ABF system. This clearly shows that use of antimicrobials at production level is associated with antimicrobial resistance. There are numerous studies that have shown such an association in different pathogens besides *Campylobacter*. Hershberger et al., recently showed that resistance in *Enterococci* was more common in swine farms that used antimicrobial agents (Hershberger et al., 2005). The use of enrofloxacin in the poultry industry has been shown to introduce FQ resistance in *C. jejuni* and *C. coli* (Humphrey et al., 2005). Although we did not find disinfectant use at the farm a risk factor for antimicrobial resistance, previous studies have shown that disinfectants can lead to activation of multidrug efflux pumps like use of cyclohexane has been shown to activate the AcrAB efflux pump inn *Salmonella* conferring resistance to multiple antimicrobials (Randall et al., 2001). Similar efflux pumps have been detected in *Campylobacter* also including the CmeABC pump that have been shown to be active even in the absence of antimicrobial pressure (Zhang et al., 2003). Eventhough antimicrobials were not used at the ABF farms; isolation of resistant strains of *C. coli* indicates the presence of other risk factors that may contribute to the dissemination of resistant strains. Antimicrobial use at farm was a risk factor for resistance to more than two antimicrobials in *C. coli*. This is concerning as there is risk of dissemination of these strains.

2.6 Conclusions

This study highlights the common occurrence of antimicrobial resistant *C.coli* from both the conventional and the ABF at farm and slaughter. High prevalence of *C. coli* observed at the farm level provides a strong potential for introduction of the pathogen at slaughter. Even though we detected higher number of resistant and MDR strains in isolates from the conventional system, the high proportion of antimicrobial resistant *C.coli* isolated from the ABF system warrants concern and points to the possible role of other environmental factors besides direct antimicrobial use in resistance development and transmission. This is the first report of isolation of fluoroquinolone resistant *C. coli* from pigs in the United States among pigs. MDR ciprofloxacin resistant *C.coli* isolates from swine especially from the ABF reared pigs is alarming since this antimicrobial is used in treatment of severe invasive cases of campylobacteriosis and severely limits its therapeutic use. Association of antimicrobial use at the conventional farms with resistance in *C .coli* highlights the role played by selective pressure on developing antimicrobial resistance. However, detection of resistance in the ABF farms where no selection pressure is present indicates the presence of other risk factors that are transmitting resistant strains to the pigs. Phenotypic heterogeneity observed indicates the presence of multiple strains of *Campylobacter* and necessitates the testing of multiple isolates from one positive animal.

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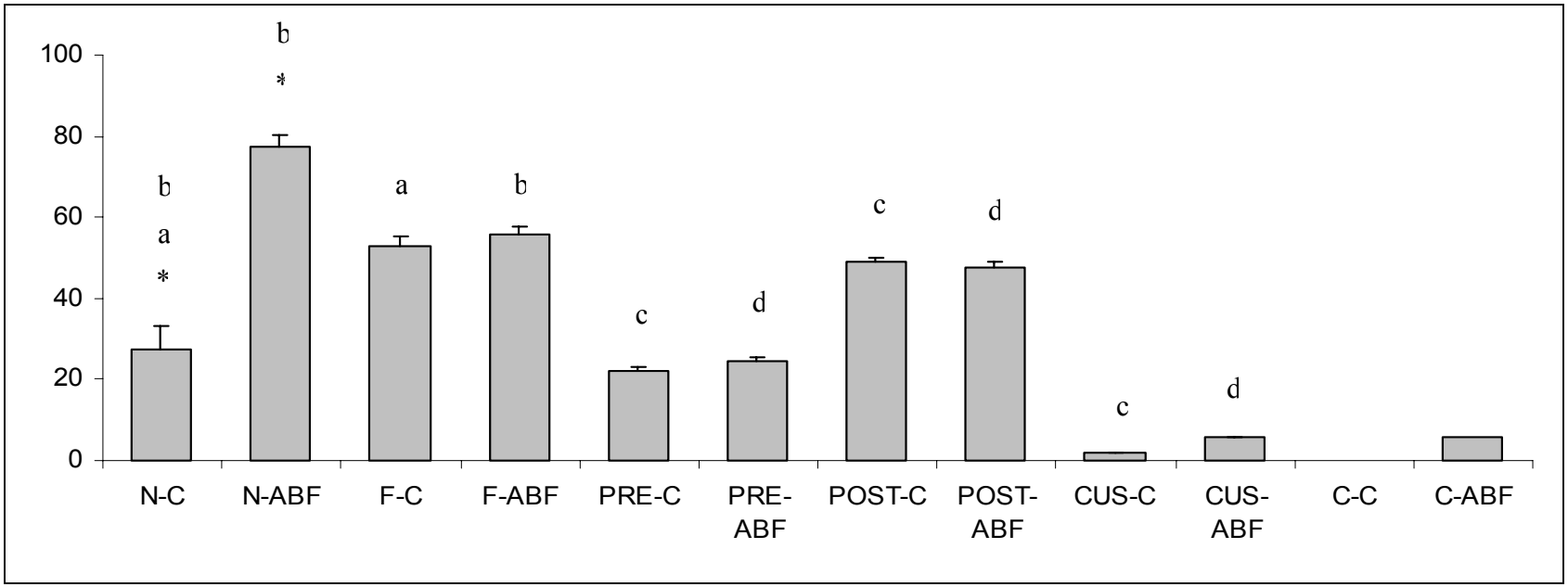


Figure 2.1 *Campylobacter coli* prevalence at the farm and slaughter level in the two production systems

Abbreviations on the X-axis with number of pigs/ carcasses sampled: N-C (Conventional Nursery, N=105); N-ABF (ABF Nursery, N=141); F-C (Conventional Finishing, N=370); F-ABF (ABF Finishing, N=292); PRE-C (Conventional Pre-Evisceration, N=103); PRE-ABF (ABF Pre-Evisceration, N=78); POST-C (Conventional Post-Evisceration, N=98); POST-ABF (ABF Post-Evisceration, N=88); CUS-C (Conventional Post-Chill USDA, N=107); CUS-ABF (ABF Post-Chill USDA, N=88); C-C (Conventional Post-Chill Single Swipe, N=108) and C-ABF (ABF Post-Chill Single Swipe, N=87).

Y-axis shows percent prevalence; $^{*, a, b, c, d}$ Bars sharing common superscripts were significantly different at $P < 0.05$

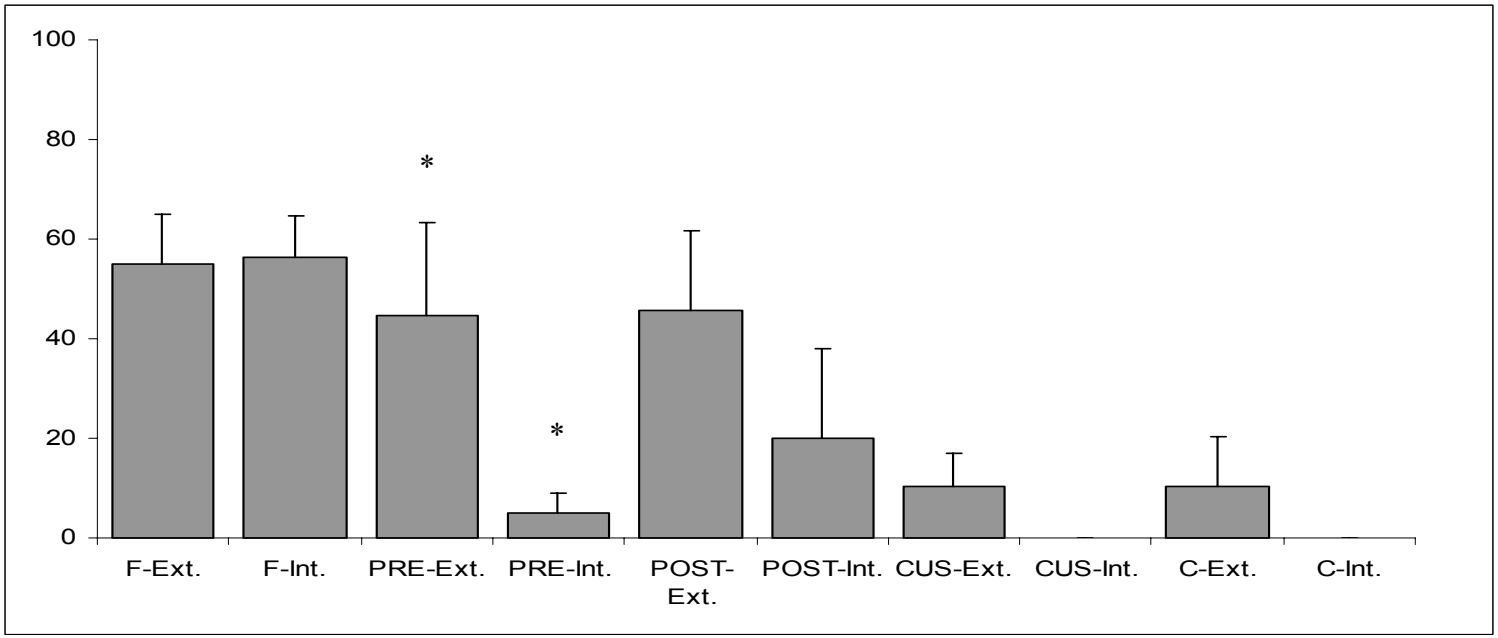


Figure 2.2 *Campylobacter coli* prevalence at the farm and slaughter level in the two antimicrobial free production systems

Abbreviations on the X-axis (n= number of pigs or carcass tested): F-Ext (Extensive Finishing, n=118); F-Int (Intensive Finishing, n=174); PRE-Ext (Extensive Pre-Evisceration, n=38); PRE-Int (Intensive Pre-Evisceration, n=40); POST-Ext (Extensive Post-Evisceration, n=48); POST-Int (Intensive Post-Evisceration, n=40); CUS-Ext (Extensive Post-Chill USDA, n=48); CUS-Int (Intensive Post-Chill USDA, n=40); C-Ext (Extensive Post-Chill Single Swipe, n=48) and C-Int (Intensive Post-Chill Single Swipe, n=39)

* Bars sharing common superscripts were significantly different at $P < 0.05$ (chi-square test)

[a,] * Bars sharing common superscripts were significantly different at $P < 0.05$ (chi-square test); Y-axis shows percent prevalence

Table 2.1 Comparison of antimicrobial resistance frequency among the *Campylobacter coli* isolates from conventional and ABF production systems at farm and slaughter

Production Stage	Production System	Isolates Tested	Chloramphenicol	Ciprofloxacin	Erythromycin	Gentamicin	Nalidixic Acid	Tetracycline
Farm	**Conventional**	450	8 (1.77) [*a]	13 (2.9) [2]	347 (77.1) [3]	4 (0.9)	31 (6.88) [b]	374 (83.11) [5]
	ABF [a]	660	9 (1.36) [a]	4 (0.6) [2]	220 (33.33) [3]	0	49 (7.42) [b]	372 (56.36) [5]
Slaughter	**Conventional**	183	8 (4.37) [1]	0	149 (81.42) [4]	1 (0.54)	2 (1.09)	146 (79.78) [6]
	ABF	166	2 (1.2) [1]	0	67 (40.36) [4]	1 (0.6)	1 (0.6)	75 (45.18) [6]
Total Isolates		1459	27 (1.85)	17 (1.16)	783 (53.66)	6 (0.41)	80 (5.48)	967 (66.27)

ABF[a]: Antimicrobial Free Farms

* Isolates with percent resistance in the brackets. For each antimicrobial, figures sharing common alphabet superscripts were not significantly different ($P > 0.05$) while figures sharing common digits in the superscripts were significantly different at $P < 0.05$

Table 2.2 Comparison of antimicrobial resistance frequency among the *Campylobacter coli* isolates from conventional and ABF production systems at different stages of production

Production Stage	Production System	Isolates Tested	Chloramphenicol	Ciprofloxacin	Erythromycin	Gentamicin	Nalidixic Acid	Tetracycline
Farm								
Nursery	**Conventional**	64	5 (7.81)	11 (17.19)	51 (79.69) [1, a]	4 (6.25)	16 (25) [16, 17]	59 (92.19) [3, 9]
	ABF	294	1 (0.34)	1 (0.34)	75 (25.51) [1, 13]	0	22 (7.48) [b, 17]	190 (64.63) [3,12]
Finishing	**Conventional**	386	3 (0.77)	2 (0.51)	296 (76.68) [2, a]	0	15 (3.88) [16, 18]	315 (81.61) [4, 9]
	ABF	366	8 (2.18)	3 (0.82)	145 (36.62) [2, 13]	0	27 (7.37) [b, 18]	182 (49.73) [4, 12]
Slaughter								
Pre-Evisceration	**Conventional**	63	2 (3.17)	0	62 (98.41) [5, 11]	0	1 (1.58)	35 (55.56) [6, 10]
	ABF	53	0	0	12 (22.64) [5, 14]	0	0	18 (33.96) [6, 15]
Post-Evisceration	**Conventional**	115	6 (5.21)	0	82 (71.3) [7, 11]	1 (0.87)	1 (0.87)	106 (92.17) [8, 10]
	ABF	95	2 (2.10)	0	49 (51.58) [7, 14]	1 (1.05)	1 (1.05)	53 (55.79) [8, 15]
Post-Chill (USDA)	**Conventional**	5	0	0	5 (100)	0	0	5 (100)
	ABF	12	0	0	3 (25)	0	0	3 (25)
Post-Chill	**Conventional**	NA	NA	NA	NA	NA	NA	NA
	ABF	6	0	0	0	0	0	1 (16.66)
Total Isolates		1459	27 (1.85)	17 (1.16)	783 (53.66)	6 (0.41)	80 (5.48)	967 (66.27)

ABF[a]: Antimicrobial Free Farms

*Isolates with percentage in the brackets. For each antimicrobial, figures sharing common alphabet superscripts were not significantly different ($P > 0.05$) while figures sharing common digits in the superscripts were significantly different at $P < 0.05$

Table 2.3 Univariate analysis of association between the outcome variable (antimicrobial resistance) with production system and processing stages

Outcome: Antimicrobial Resistance	Exposure variable	Number positive (%)		OR value	95% CI	Chi-Square *P*- value [a]
		Conventional (Isolates = 633)	ABF (Isolates = 826)			
Erythromycin	**Production System**	496 (78.3)	287 (34.7)	6.7	5.36-8.61	0
Tetracycline		520 (82)	447(54)	3.6	1.41-1.61	0
Chloramphenicol		16 (2.5)	11(1.3)	1.9	0.90-40.9	0.093
Nalidixic Acid		33 (5.2)	50 (6)	0.8	0.54-1.33	0.49
Ciprofloxacin		13 (2)	4 (0.4)	4.3	1.47-12.6	0.006
		Conventional Farm (Isolates = 450)	**ABF Farm (Isolates = 660)**			
Erythromycin	**Production Stage (Farm Level)**	347 (77.1)	220 (33.3)	6.7	5.12-8.85	0
Tetracycline		374 (83.1)	372 (56.3)	3.8	2.8-5.09	0
Chloramphenicol		8 (1.7)	9 (1.3)	1.3	0.51-3.31	0.58
Nalidixic Acid		31 (6.8)	49 (7.4)	0.92	0.58-1.46	0.73
Ciprofloxacin		13 (2.8)	4 (0.6)	4.86	1.65-14.25	0.002
		Conventional Slaughter (Isolates = 183)	**ABF Slaughter (Isolates = 166)**			
Erythromycin	**Production Stage (Slaughter Level)**	149 (81.4)	67 (40.3)	6.47	3.99-10.49	0
Tetracycline		146 (79.7)	75 945.1)	4.78	2.98-7.67	0
Chloramphenicol		8 (4.3)	2 (1.2)	3.81	0.90-16.07	0.07

[a] $P < 0.05$ was considered statistically significant

Table 2.4 Comparison of antimicrobial resistance frequency among the *Campylobacter coli* isolates from Intensive and Extensive reared ABF pigs at different stages of production

	Number of isolates resistant to antimicrobials (Percent)*							
Production Stage	**ABF[a] Production System Type**	**Isolates Tested**	**Chloramphenicol**	**Ciprofloxacin**	**Erythromycin**	**Gentamicin**	**Nalidixic Acid**	**Tetracycline**
Farm								
Finishing	**Extensive**	162	1 (0.6)	2 (1.2)	32 (19.7)[2, 5]	-	5 (3)	62 (38.2)[1, 7]
	Intensive	204	7 (3.4)	1 (0.5)	113 (55.3)[3, 5]	-	22 (10.7)	120 (58.8)[a, 7]
Slaughter								
Pre-Evisceration	**Extensive**	47	-	-	11 (23.49)[8]	-	-	15 (31.9)[b]
	Intensive	6	-	-	1 (16.6)	-	-	3 (50)
Post-Evisceration	**Extensive**	52	1 (1.9)	-	22 (42.3)[8]	1 (1.9)	-	15 (28.8)[b]
	Intensive	43	1 (2.3)	-	27 (62.7)	-	1 (2.3)	38 (88.3)
Post-Chill (USDA)	**Extensive**	12	-	-	3 (25)	-	-	3 (25)
	Intensive	-	-	-	-	-	-	-
Post-Chill	**Extensive**	6	-	-	-	-	-	1 (16.6)
	Intensive	-	-	-	-	-	-	-
Total Isolates		532	10 (1.8)	3 (0.5)	209 (39.2)	1 (0.1)	28 (5.2)	257 (48.3)

[a] Antimicrobial Free Farms

* Isolates with percentage in the brackets. For each antimicrobial, figures sharing common alphabet superscripts were not significantly different ($P > 0.05$) while figures sharing common digits in the superscripts were significantly different at $P < 0.05$ (chi-square test and Fisher's exact two-tailed)

Table 2.5 Antimicrobial resistance pattern observed among *Campylobacter coli* isolates in the two production systems

Antimicrobial Resistance Patterns [b,f]	Farm Level		Slaughter Level	
	Conv.[c]	ABF[d]	Conv.	ABF
Pansusceptible	27 (6) [e]	213 (32.2)	6 (3.2)	58 (34.9)
Ch Ery [a]	-	3 (0.4)	-	-
Ery Tet	264 (58.6)	122 (18.7)	111 (60.6)	38 (22.8)
Nal Tet	3 (0.6)	18 (2.7)	-	-
Ch Ery Tet	2 (0.4)	3 (0.4)	10 (5.4)	-
Cip Nal Tet	-	4 (0.6)	-	-
Ery Nal Tet	15 (3.3)	25 (3.7)	-	-
Cip Ery Nal Tet	10 (2.2)	1 (0.1)		
Ch Cip Ery Nal Tet	2 (0.4)	-	-	-
Ery Gen Tet	3 (0.6)	-	-	-
Ery Nal	3 (0.6)	-	-	-

[a] Ch, Chloramphenicol; Cip, Ciprofloxacin; Ery, Erythromycin; Gen, Gentamicin; Nal, Nalidixic Acid; Tet, Tetracycline

[b] Resistance patterns not shown in the table include single *C. coli* isolate each of Cip Ery Gen Nal Tet (Conventional Farm), Ch Ery Nal and Cip Gen Nal Tet (Conventional Slaughter), Ch Ery Gen and Ch Ery Nal Tet (ABF Slaughter). Number of isolates tested per production system is given in Table 2.2.

[c] Conventional Farms; [d] Antimicrobial Free Farms; [e] Number (%) of *C. coli* isolates showing the resistance pattern

[f] Resistance against individual antimicrobials is shown in Table 2.1

Table 2.6 Univariate analysis of association between the outcome variable (Pansusceptibility and antimicrobial resistance patterns) with the two production systems

Outcome: Pansusceptibility and Antimicrobial Pattern	Exposure variable	Number positive (%)		OR value	95% CI
		Conventional (Isolates = 633)	ABF (Isolates = 826)		
Pansusceptible	**Production System**	33 (5.2)	271 (32.8)	0.11	0.07-0.16
Ery Tet		375 (59.2)	160 (19.3)	6	4.7-7.6
Ery Nal Tet		15 (2.3)	25 (3)	0.77	0.41-1.47
		Conventional Farm (Isolates = 450)	**ABF Farm (Isolates = 660)**		
Pansusceptible	**Production Stage (Farm Level)**	27 (6)	213 (32.2)	0.13	0.08-0.2
Ery Tet		264 (58.6)	122 (18.7)	8.19	6.26-10.7
Ery Nal Tet		15 (3.3)	25 (3.7)	1.1	0.58-2.09
		Conventional Slaughter (Isolates = 183)	**ABF Slaughter (Isolates = 166)**		
Pansusceptible	**Production Stage (Slaughter Level)**	6 (3.2)	58 (34.9)	0.06	0.02-0.14
Ery Tet		111 (60.6)	38 (22.8)	5.19	3.25-8.28

[a] $P < 0.05$ was considered statistically significant

Table 2.7 Antimicrobial resistance pattern observed among *Campylobacter coli* isolates in the two production systems at the processing level

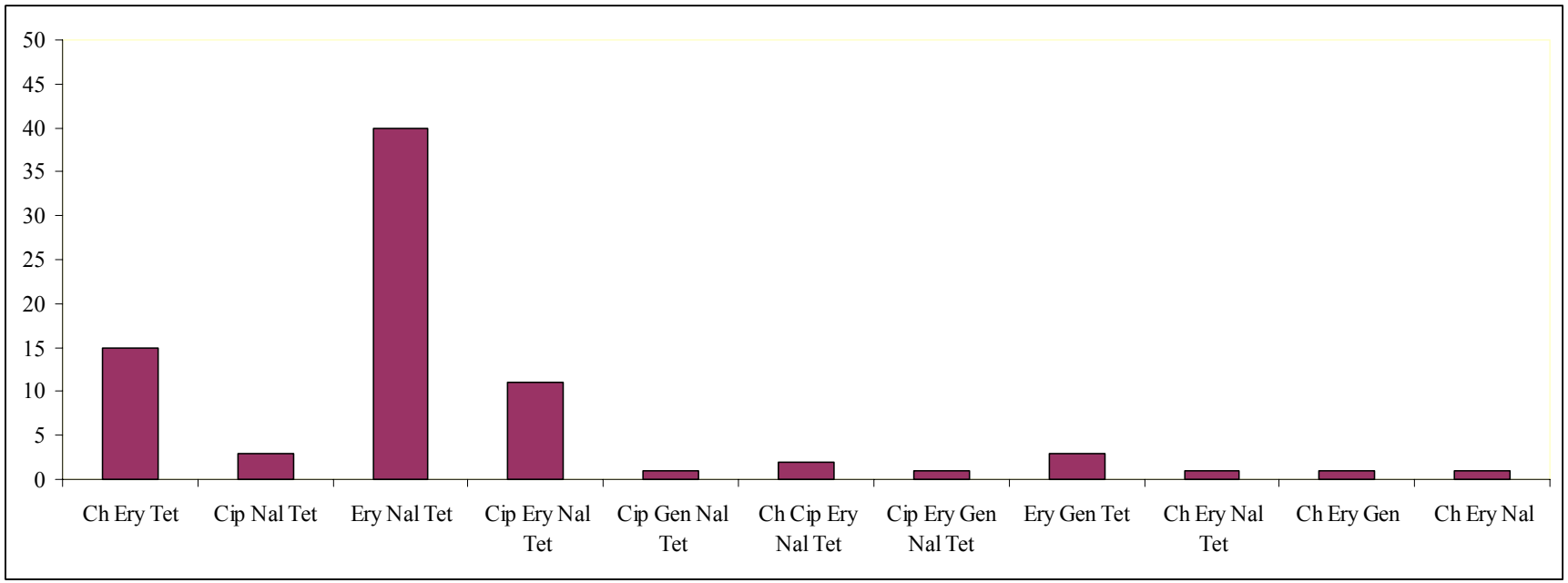
Antimicrobial Resistance Patterns [b,f]	Production System and Processing Stage									
	Farm				Slaughter					
	Nursery		Finishing		Pre-Evisceration		Post-Evisceration		Post-Chill (USDA)	
	Conv.[c]	ABF[d]	Conv.	ABF	Conv.	ABF	Conv.	ABF	Conv.	ABF
Pansusceptible	1 (1.6) [e]	81 (27.5)	26 (6.7)	132 (35.6)	1 (1.5)	28 (51.9)	5 (4.3)	23 (23.5)	-	7 (58.3)
Ch Ery [a]	-	-	-	3 (0.8)	-	-	-	-	-	-
Ery Tet	30 (47)	44 (15)	234 (60.6)	78 (21)	34 (52.3)	5 (9.3)	72 (62.6)	32 (32.7)	5 (100)	1 (8.3)
Nal Tet	1 (1.6)	10 (3.4)	2 (0.5)	8 (2.2)	-	-	-	-	-	-
Ch Ery Tet	1 (1.6)	1 (0.3)	1 (0.3)	2 (0.5)	3 (4.7)	-	7 (6)	-	-	-
Cip Nal Tet	-	1 (0.3)	-	3 (0.8)	-	-	-	-	-	-
Ery Nal Tet	3 (4.7)	9 (3)	12 (3.1)	16 (4.3)	-	-	-	-	-	-
Cip Ery Nal Tet	10 (15.6)	-	-	1 (0.3)	-	-	-	-	-	-
Ch Cip Ery Nal Tet	-	-	2 (0.5)	-	-	-	-	-	-	-
Ery Gen Tet	3 (4.7)	-	-	-	-	-	-	-	-	-
Ery Nal	-	1 (0.3)	-	2 (0.5)	-	-	-	-	-	-

[a] Ch, Chloramphenicol; Cip, Ciprofloxacin; Ery, Erythromycin; Gen, Gentamicin; Nal, Nalidixic Acid; Tet, Tetracycline

[b] Resistance patterns not shown in the table include single *C. coli* isolate each of Cip Ery Gen Nal Tet (Conventional; Nursery), Ch Ery Nal (Conventional; Pre-evisceration), Cip Gen Nal Tet (Conventional; Post-evisceration), Ch Ery Gen (ABF; Post-evisceration) and Ch Ery Nal Tet (ABF; Post-evisceration). Number of isolates tested per production system is given in Table 1.

[c] Conventional Farms; [d] Antimicrobial Free Farms; [e] Number (%) of *C. coli* isolates showing the resistance pattern

[f] Resistance against individual antimicrobials is shown in Table 2.1



X- axis represents the different multidrug resistant patterns observed in the two production systems

Y-axis represents the percent isolates

Figure 2.3 Graph representing the frequency of 11 different multidrug resistant *Campylobacter coli* isolates from the two production systems

Table 2.8 *Campylobacter coli* antimicrobial resistance patterns comparison between the Intensive and the Extensive ABF production system at the farm and slaughter levels

Antimicrobial [f] Resistance Pattern	Production Stage							
	Farm [d]		Slaughter [e]					
	Finishing		Pre-Evisceration		Post-Evisceration		Post-Chill (USDA)	Post-Chill
	Extensive	Intensive	Extensive	Intensive	Extensive	Intensive	Extensive	Intensive
Pansusceptible	84 (51.8)*	46 (22.5)	26 (54.2)	2 (40)	22 (41.5)	1 (2.3)	7 (58.3)	-
Ch Ery	1 (0.6)	2 (1)	-	-	-	-	-	-
Ery Tet	15 (9.2)	61 (30)	4 (8.3)	1 (20)	7 (13.2)	25 (59.5)	1 (8.3)	-
Nal Tet	1 (0.6)	7 (3.4)	-	-	-	-	-	-
Ch Ery Tet	-	2 (1)	-	-	-	-	-	-
Cip Nal Tet	2 (1.2)	-	-	-	-	-	-	-
Ery Nal Tet	-	16 (7.8)	-	-	-	-	-	-
Cip Ery Nal Tet	-	1 (0.5)	-	-	-	-	-	-
Ery Nal	-	2 (1)	-	-	-	-	-	-
Ch Ery Nal Tet	-	-	-	-	-	1 (2.3)	-	-
Ch Ery Gen	-	-	-	-	1 (1.8)	-	-	-

[a] Antimicrobial Free Farms ,* Isolates with percentage in the brackets.

[b] Ch, Chloramphenicol; Cip, Ciprofloxacin; Ery, Erythromycin; Gen, Gentamicin; Nal, Nalidixic Acid; Tet, Tetracycline

[d] Number of isolates at the farm level: 162 (Extensive ABF) and 204 (Intensive ABF)

[e] Number of isolates tested at the Slaughter level: 119 (Extensive ABF) and 47 (Intensive ABF)

[f] Resistance against individual antimicrobials is shown in Table 2.4

Table 2.9 MIC data and antimicrobial resistance profile of *Campylobacter coli* isolates from the two production systems at different stages of production

			Number of isolates (%) with MIC (mg/L)											
Antimic Agents	**Production Stage**	**Production System**	**≤0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥32**
Ch [f]	**Farm**	**Conventional**				2 (0.4)	7 (1.5)	3 (0.6)	89 (19.7)	246 (54.6)	76 (16.8)	19 (4.2)	7 (1.5)	1 (0.2)
(0.25-128)		**ABF**					7 (1)	7 (1)	82 (12.4)	314 (47.5)	232 (35)	10 (1.5)	6 (0.9)	3 (0.4)
	Slaughter	**Conventional**						2 (1)	36 (19.4)	118 (64.48)	16 (8.74)	3 (1.6)	1 (0.5)	7 (3.8)
		ABF				1 (0.6)	4 (2.4)	4 (2.4)	27 (16.26)	110 (66.26)	18 (10.84)		1 (0.6)	1 (0.6)
Cip	**Farm**	**Conventional**	11 (2.4)	60 (13.3)	180 (40)	157 (34.8)	24 (5.3)	3 (0.6)	2 (0.4)	13 (2.8)				
(0.008-4)		**ABF**	15(2.7)	72 (10.9)	271 (41.06)	186 (28.18)	75 (11.36)	20 (3)	17 (2.57)	4 (0.6)				
	Slaughter	**Conventional**	3 (1.6)	29 (15.84)	73 (39.89)	72 (39.34)	1 (0.5)	5 (2.73)						
		ABF	12(7.8)	74 (44.57)	68 (40.96)	5 (3)	5 (3)	2 (1.2)						
Ery	**Farm**	**Conventional**		1 (0.2)		1 (0.2)	5 (1.1)	19 (4.2)	30 (6.7)	47 (10.4)	16 (3.5)	7 (1.5)	14 (3.1)	310 (68.9)
(0.06-32)		**ABF**		2 (0.3)		15 (2.27)	36 (5.4)	93 (14)	187 (28.3)	102 (15.3)	38 (5.8)	20 (3.1)	15 (2.2)	147 (22.27)
	Slaughter	**Conventional**					3 (1.6)	6 (3.2)	14 (7.6)	12 (6.5)	6 (3.27)	5 (2.7)	6 (3.27)	127 (69.39)
		ABF			2 (1.2)	1 (0.6)	10 (6)	19 (11.44)	46 (27.7)	23 (13.85)	20 (12)	9 (5.42)	4 (2.4)	28 (16.86)
Gen	**Farm**	**Conventional**		3 (0.6)	2 (0.4)	6 (1.3)	97 (21.5)	226 (50)	103 (22.9)	6 (1.3)	2 (0.4)	2 (0.4)		2 (0.4)
(0.06-32)		**ABF**		2 (0.3)	7 (1)	14 (2)	116 (17.57)	317 (48)	180 (27.27)	21 (3.18)	3 (0.45)			
	Slaughter	**Conventional**				11 (6)	50 (27.32)	104 (56.83)	17 (9.28)				1 (0.5)	
		ABF			1 (0.6)	2 (1.2)	57 (34.33)	77 (46.38)	27 (16.26)		1 (0.6)	1 (0.6)		
Nal	**Farm**	**Conventional**						3 (0.6)	25 (5.5)	122 (27.1)	217 (48.2)	50 (11.1)	9 (2)	22 (4.8)
(0.25-128)		**ABF**				3 (0.45)	2 (0.3)		43 (6.51)	208 (31.51)	291 (44)	59 (8.93)	15 (2.27)	34 (5.1)
	Slaughter	**Conventional**							17 (9.28)	80 (43.71)	71 (38.79)	12 (6.55)	1 (0.5)	1 (0.5)
		ABF				1 (0.6)	1 (0.6)	1 (0.6)	27 (16.26)	96 (57.83)	34 (20.48)	5 (3)		1 (0.6)
Tet	**Farm**	**Conventional**			2 (0.4)	6 (1.3)	3 (0.6)	5 (1.1)	15 (3.3)	2 (0.4)	42 (9.3)	30 (6.7)	98 (21.8)	246 (54.9)
(0.06-32)		**ABF**		4 (0.6)	12 (1.8)	31 (4.69)	31 (4.69)	34 (5.15)	76 (11.51)	55 (8.33)	45 (6.81)	45 (6.81)	78 (11.8)	249 (37.72)
	Slaughter	**Conventional**				1 (0.5)	1 (0.5)			23 (12.56)	12 (6.55)	9 (4.9)	31 (16.93)	106 (57.92)
		ABF			11 (6.62)	7 (4.21)	12 (7.22)	3 (1.8)	7 (4.21)	35 (21)	16 (9.63)	6 (3.61)	32 (19.27)	37 (22.28)

[a] Antimicrobial Free Farms

[b] Dilution range based on the approved NCCLS Standards for *Campylobacter*

[c] Breakpoint level based on *C.jejuni* ATCC 33560. Columns in bold also indicate the breakpoint level for each antimicrobial.

[d] Number of *C. coli* isolates at the farm level: 450 (Conventional) and 660 (ABF)

[e] Number of *C. coli* isolates at the Slaughter level: 183 (Conventional) and 166 (ABF)

[f] Ch, Chloramphenicol; Cip, Ciprofloxacin; Ery, Erythromycin; Gen, Gentamicin; Nal, Nalidixic Acid; Tet, Tetracycline

Table 2.10 MIC data and antimicrobial resistance profile of *Campylobacter coli* isolates from the extensive and intensive ABF production systems at farm and slaughter

			Number of isolates (%) with MIC (mg/L)											
Antimicrobial Agents	**Production Stage**	**ABF**	**≤0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥32**
Ch [f]	**Farm**	**Extensive**					5 (3)	3 (1.8)	31 (19)	101 (62.3)	31 (19)			1 (0.6)
(0.25-128)		**Intensive**					2 (0.9)	4 (1.9)	47 (23)	79 (38.7)	59 (28.9)	7 (3.4)	5 (2.4)	1 (0.5)
	Slaughter	**Extensive**				1 (0.8)			9 (7.7)	90 (76.92)	16 (13.67)		1 (0.8)	
		Intensive					4 (8.16)	4 (8.16)	19 (38.7)	19 (38.8)	2 (4)			1 (2)
Cip	**Farm**	**Extensive**	7 (6.1)	23 (14)	109 (67.3)	16 (9.8)	1 (0.6)	3 (1.8)	1 (0.6)	2 (1.2)				
(0.008-4)		**Intensive**	3 (1.4)	26 (12.7)	109 (53.4)	31 (15)	15 (7.3)	5 (2.4)	14 (6.8)	1 (0.5)				
	Slaughter	**Extensive**	6(5)	63 (53.84)	42 (35.89)	2 (1.7)	3 (2.5)	1 (0.8)						
		Intensive	8 (18.3)	13 (26.5)	23 (47)	3 (6.1)	2 (4)							
Ery	**Farm**	**Extensive**		2 (1.2)		4 (2.5)	18 (11)	31 (19)	51 (31.5)	24 (14.8)	14 (8.6)	6 (3.7)		12 (7.4)
(0.06-32)		**Intensive**				2 (0.9)	10 (4.9)	10 (4.9)	32 (15.6)	32 (15.6)	3 (1.4)	10 (4.9)	9 (4.4)	96 (47)
	Slaughter	**Extensive**					10 (8.54)	17 (14.52)	30 (25.64)	23 (19.65)	13 (11.11)	7 (5.98)	1 (0.8)	12 (10.25)
		Intensive			2 (4)	1 (2)			15 (30)	1 (2)	4 (8.2)	1 (2)	3 (6.1)	20 (44.9)
Gen	**Farm**	**Extensive**		2 (1.2)	5 (3)	4 (2.5)	38 (23.4)	99 (61)	11 (6.8)	1 (0.6)	2 (1.2)			
(0.06-32)		**Intensive**				7 (3.4)	48 (23.5)	123 (6.)	25 (12.2)	1 (0.5)				
	Slaughter	**Extensive**				1 (0.8)	37 (31.62)	51 (43.58)	27 (23)			1 (0.8)		
		Intensive			1 (2)	1 (2)	20 (40.8)	25 (51)	1 (2)		1 (2)			
Nal Acid	**Farm**	**Extensive**				3 (1.8)	2 (1.2)		4 (2.5)	114 (70)	29 (17.9)	5 (3)	1 (0.6)	4 (2.5)
(0.25-128)		**Intensive**							16 (7.8)	60 (29.4)	92 (45)	9 (4.4)		27 (13.2)
	Slaughter	**Extensive**						1 (0.8)	20 (17)	75 (64.1)	19 (16.23)	2 (1.7)		
		Intensive				1 (2)	1 (2)		9 (18.4)	18 (36.7)	16 (32.6)	3 (6.1)		1 (2)
Tet	**Farm**	**Extensive**		4 (2.5)	8 (5)	16 (9.8)	17 (10.5)	15 (9.2)	16 (9.8)	17 (10.5)	8 (5)	13 (8)	27 (16.6)	21 (13)
(0.06-32)		**Intensive**				11 (5.4)	10 (4.9)	2 (0.9)	28 (13.7)	4 (1.9)	29 (14.2)	17 (8.3)	31 (15)	72 (35.3)
	Slaughter	**Extensive**			10 (8.54)	5 (4.27)	12 (10.25)	2 (1.7)	7 (5.98)	30 (25.64)	15 (12.82)	5 (4.27)	13 (11.11)	16 (13.67)
		Intensive			1 (2)	2 (4)		1 (2)			2 (4)	1 (2)	19 (42.8)	21 (42.8)

[a] Antimicrobial Free Farms

[b] Dilution range based on the approved NCCLS Standards for *Campylobacter*

[c] Breakpoint level based on *C.jejuni* ATCC 33560. Columns in bold also indicate the breakpoint level for each antimicrobial.

[d] Number of *C. coli* isolates at the farm level: 162 (Extensive) and 204 (Intensive)

[e] Number of *C. coli* isolates at the Slaughter level:117 (Extensive) and 49 (Intensive)

[f] Ch, Chloramphenicol; Cip, Ciprofloxacin; Ery, Erythromycin; Gen, Gentamicin; Nal, Nalidixic Acid; Tet, Tetracycline

Table 2.11 Phenotypic diversity (Heterogeneity) in the MIC levels and the resistance patterns of *Campylobacter coli* in the two production systems at farm and slaughter

Production Stage	Production System	Pigs/Carcass Positive	Heterogeneity		
			Based on MIC		Based on resistance pattern
			MIC (2-Fold difference)	MIC (4-Fold difference)	Resistance pattern
Farm	**Conventional**	225	121 (53.8) [b]	75 (33.3)	60 (26.7)
	ABF [a]	272	169 (62)	117 (43)	102 (37.5)
Slaughter	**Conventional**	73	47 (64.4)	30 (41)	25 (34.2)
	ABF	71	51 (71.8)	28 (39.4)	19 (26.8)
Total Isolates		641	388 (60.5)	250 (39)	206 (32)

[a] Antimicrobial Free Farms

[b] Number of isolates with different MIC (percent)

Table 2.12 Antimicrobials and disinfectant use at farm level in the conventional production system

Farm Number	Production Stage	Antimicrobials Used	Administration Route	Chemical Disinfectant
1	Nursery	CSP 250, OTC [a], Tylan	Feed	Used only at Finishing
	Nursery	Penicillin, Naxel	Injectible	
	Finishing	OTC, Tylan	Feed	
2	Nursery	CSP 250, OTC, Tylan	Feed	Used at both Nursery and Finishing
	Nursery	Penicillin, Naxel	Injectible	
	Finishing	OTC, Tylan	Feed	
3	Finishing	OTC, Tylan	Feed	Used only at Finishing
	Finishing	Penicillin, Naxel	Injectible	
4	Finishing	OTC, Tylan	Feed	Used only at Finishing
	Finishing	Penicillin, Naxel	Injectible	
5	Finishing	OTC, Tylan	Feed	Used only at Finishing
	Finishing	Penicillin	Injectible	
6	Nursery	CSP 250, OTC, Tylan	Feed	Used only at Finishing
	Finishing	Penicillin	Injectible	
	Finishing	OTC, Tylan	Feed	
7	Nursery	Tylan	Feed	Used at both Nursery and Finishing
	Nursery	Penicillin, Naxel	Injectible	
	Finishing	OTC, Tylan	Feed	
	Finishing	Penicillin	Injectible	
8	Nursery	Tylan	Feed	Used at both Nursery and Finishing
	Nursery	Penicillin, Naxel	Injectible	
	Finishing	OTC, Tylan	Feed	
	Finishing	Penicillin	Injectible	
9	Nursery	OTC, Tylan, Aureomycin 50	Feed	Used at Nursery only
	Nursery	Lincomix 50, Tylan 50,	Injectible	
	Finishing	Aureomycin 50, BMD 30	Feed	
	Finishing	Lincomix 200, Tylan 200	Injectible	

[a,] Oxytetracycline

Table 2.13 Univariate analysis of the risk factors with the outcome of interest (resistance to antimicrobials)

Risk Factors	Outcome of interest		
	Erythromycin resistance	Tetracycline resistance	Resistance to 2 or more antimicrobials
	P-value [a]	*P*-value	*P*-value
Production system	< 0.001	< 0.001	< 0.001
Penicillin injection	< 0.001	< 0.001	< 0.001
Naxel injection	< 0.001	< 0.001	< 0.001
Oxytetracycline in feed	< 0.001	< 0.001	< 0.001
Tylan in feed	< 0.001	< 0.001	< 0.001
Chemical Disinfectant	0.23	= 0.17	= 0.01

[a] $P < 0.25$ was considered statistically significant

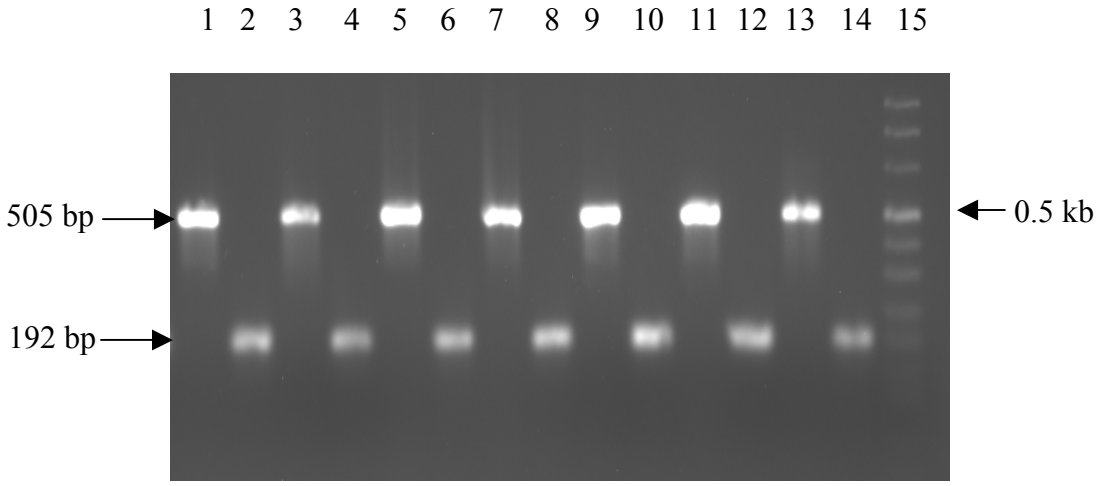
Table 2.14 Final multivariate model for the three outcomes (Erythromycin resistance, tetracycline resistance and resistance to two or more antimicrobials simultaneously)

Risk Factor	Outcome of interest					
	Model A (Erythromycin Resistance)		Model B (Tetracycline Resistance)		Model C (Resistance to 2 or more antimicrobials)	
	OR	95% CI	OR	95% CI	OR	95% CI
Conventional Production system	2.39	1.5-3.6	5.1	3.2-8.1	2.3	1.56-3.37

[a] $P < 0.05$ was considered statistically significant

Table 2.15 Minimum Inhibitory Concentration, Mismatch Amplification Mutation Assay PCR and Sequencing of QRDR region results for the ciprofloxacin resistant *Campylobacter coli* isolates

Isolate ID	Production System	Processing Stage	MIC against Ciprofloxacin (mg/L)	MAMA PCR (Thr-86- Ile Mutation)	Other mutations detected by sequencing
260	ABF	Nursery	8	Negative	
3804	ABF	Finishing	4	Negative	
4075	ABF	Finishing	8	Negative	
4076	ABF	Finishing	8	Negative	
461	Conventional	Nursery	4	Negative	
475	Conventional	Nursery	> 4	Positive	
524	Conventional	Nursery	> 4	Positive	
525	Conventional	Nursery	64	Positive	
526	Conventional	Nursery	64	Positive	
548	Conventional	Nursery	> 64	Positive	
549	Conventional	Nursery	> 64	Positive	
552	Conventional	Nursery	> 64	Positive	
554	Conventional	Nursery	> 64	Positive	Glu-147-Asp, Silent mutation at position 116
555	Conventional	Nursery	> 64	Positive	
556	Conventional	Nursery	> 4	Positive	Silent mutation at position 108
4516	Conventional	Finishing	4	Negative	
4517	Conventional	Finishing	4	Negative	



Lanes

All isolates are loaded in pairs showing the 505 bp band positive control showing the *gyrA* gene of all the C. coli isolates. The 192 bp band indicates the Threonine to Isoleucine mutation at position 86 (ACT → ATT)

1 & 2. Isolate ID 555

3 & 4. Isolate ID 552

5 & 6. Isolate ID 526

7 & 8. Isolate ID 554

9 & 10. Isolate ID 525

11 & 12. Isolate ID 524

13 & 14. Isolate ID 475

15. Molecular Marker

Figure 2.4 PCR product gel electrophoresis image showing the quinolone resistance determining region (QRDR) and the Thr-86-Ile mutation

```
3804          TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 112
4075          TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 110
AF092101      TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 115
549           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 108
4076          TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 110
4516          TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAAATCTGCTCG-TAT 97
556           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCGCTAT 106
524           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 106
525           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 107
548           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 106
260           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 104
461           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 106
526           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 104
475           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCG-TAT 104
555           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCGCTAT 104
554           TGCTATGTAATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCGCTAT 107
552           TGCTATG-AATGATCTTGGCGTAGGAAGTAGAAGTGCATATAAAAAA-TCTGCTCGCTAT 107
              ******* ************************************** ******** ***

3804          AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATACTGCTGTTTACGATGCC- 170
4075          AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATACTGCTGTTTACGATGCC- 168
AF092101      AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATACTGCTGTTTACGATGCC- 173
549           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 166
4076          AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATACTGCTGTTTACGATGCC- 168
4516          AGTAGGGGGATGTTATCGGTAAGTATCATCCACATGGCGATACTGCTGTTTACGATGCCC 157
556           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 164
524           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 164
525           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 165
548           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 164
260           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATACTGCTGTTTACGATGCC- 162
461           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATACTGCTGTTTACGATGCC- 164
526           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 162
475           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 162
555           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 162
554           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 165
552           AGTAGGGG-ATGTTATCGGTAAGTATCATCCACATGGCGATATTGCTGTTTACGATGCC- 165
              ******** ************************************ ***************
```

Threonine to Isoleucine point mutation at position 86

```
3804          TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 230
4075          TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 228
AF092101      TTAGTAAGAATGGCACAAGATTTTTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 233
549           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 226
4076          TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 228
4516          TTAGTAAGAATGGCACAAGATTTCTTTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 217
556           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGCCAAGGAAAC 224
```

Silent mutation for Glycine at amino acid position 108

```
524           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 224
525           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 225
548           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 224
260           TTAGTAAGAATGGCACAAGATTTTTCTATGCGTTATCCAAGTATC GGACAAGGAAACAC 222
461           TTAGTAAGAATGGCACAAGATTTTTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 224
526           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 222
475           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 222
555           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 222
554           TTAGTAAGAATGGCTCAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 225
552           TTAGTAAGAATGGCACAAGATTTCTCTATGCGTTATCCAAGTATCGATGGACAAGGAAAC 225
              ************** ************************************ *********
```

Silent mutation for Alanine at amino acid position 116

```
3804          TTTGGTTCTATCGATGGTGATGGCGCTGCTGCAATGCGTTATACTGAAGCTAGAATGACA 290
4075          TTTGGTTCTATCGATGGTGATGGCGCTGCTGCAATGCGTTATACTGAAGCTAGAATGACA 288
AF092101      TTTGGTTCTATCGATGGTGATGGCGCTGCTGCAATGCGTTATACTGAAGCTAGAATGACA 293
549           TTTGGTTCTATCGATGGTGATGGCGCTGCTGCAATGCGTTATACTGAAGCTAGAATGACA 286
4076          TTTGGTTCTATCGATGGTGATGGCGCTGCTGCAATGCGTTATACTGAAGCTAGAATGACA 288
4516          TTTGGTTCTATCGATGGTGATGGCGCTGCTGCAATGCGTTATACTGAAGCTAGAATGACA 277
```

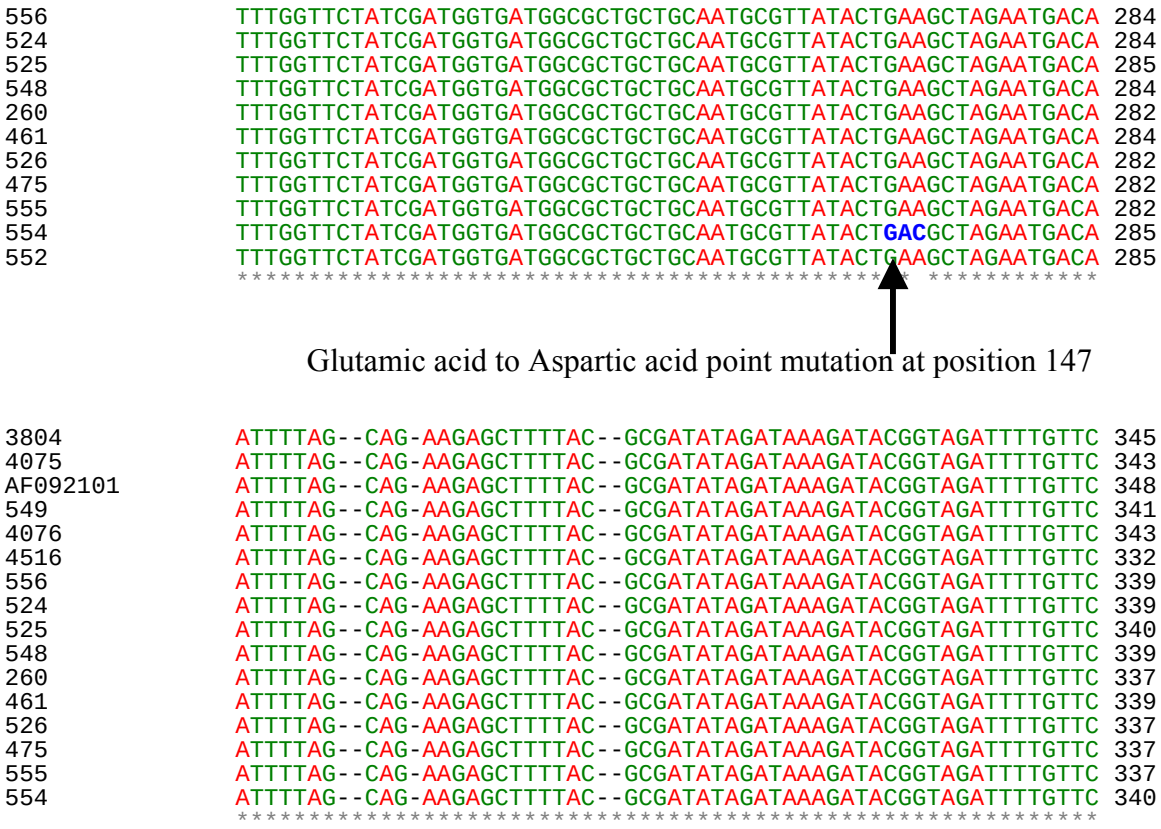


Figure 2.5 Multiple nucleotide alignment of the *gyrA* quinolone resistance determining region (QRDR) of 16 ciprofloxacin resistant *Campylobacter coli* isolates

3. *Campylobacter coli* in Swine Production: Molecular Epidemiology and Comparison of MLST and PFGE for Genotyping.

Abstract

The aim of this study was to evaluate and compare the use of two genotyping methods and determines the molecular epidemiology of *Campylobacter coli* of porcine origin. A total of 100 *C. coli* isolate from swine reared in conventional and antimicrobial free farms (ABF) and samples from slaughterhouse were genotyped using two high-resolution fingerprinting approaches: multilocus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE). Evaluation of the methods was based on their discriminatory index (DI), high throughputness, and cost and turnaround time. Index of association (I_A) was also calculated to determine the clonal structure of *C. coli*. Both methods were found to have high discriminatory power although MLST had a higher discrimination index (DI) of 0.936 than PFGE (DI= 0.889). It also had a higher throughput than PFGE. Isolates were clustered into 27 groups by MLST compared to 11 by PFGE. MLST was able to further discriminate the isolates grouped under the same cluster by PFGE. Out of the 65 MLST sequence types (ST) identified among the total isolates, 50 were reported for the first time. STs were found specific to the farm (n=38) and slaughter (n=22) with a unique clone, ST-1413, found associated only with isolates from the ABF production system exhibiting the Cip Ery Nal Tet antimicrobial resistance pattern. The weak clonal structure of the *C. coli* population among swine was further highlighted by the I_A value of 0.293. The findings of this study indicate that the use of

MLST for typing *C. coli* will allow us to better understand the molecular epidemiology of this important pathogen.

3.1 Introduction

It is important to use typing methods that have high discriminatory power to identify and differentiate sources of pathogens in animals, humans and the environment. Many phenotypic and genotypic methods have been developed for *Campylobacter*. Multilocus sequence typing (MLST) is one such genotypic method that is based on indexing the genetic variation in housekeeping genes (Dingle et al., 2001). This technique has been successfully employed for studying the longitudinal epidemiology of *C. jejuni* in humans, animals and environmental samples (Colles et al., 2003). Recently, the standardization of MLST typing method was extended to *C. coli* (Dingle et al., 2001). However, no molecular epidemiological study has been reported using MLST for *C. coli*. Pulsed field gel electrophoresis (PFGE) is another genotyping method that has been used for investigating *C. jejuni* outbreaks and genotyping *C. coli* (On, 1998; Hume et al., 2002; Sails et al., 2003; Michaud et al., 2005). Although this method is highly discriminatory, interlaboratory comparisons could be difficult due to complex protocols and accessibility to equipment and software for analyzing the patterns in multiple laboratories.

Prompted by the importance of *C. coli* as a foodborne pathogen and the paucity of molecular epidemiological information for pigs, we conducted this study. The aim was to evaluate the use of MLST and PFGE methods on isolates from swine using discriminatory power analysis, throughput, and cost, to determine the clonal structure of

C. coli by using index of association (I_A) measure and determine the genotypic diversity of *C. coli* in swine at farm and slaughter.

3.2 Materials and Methods

3.2.1 Origin of *Campylobacter coli* strains

A total of 100 isolates were selected systematically to represent production type and the processing stage from 1459 *C. coli* isolates that were isolated as a part of a cross-sectional study conducted on swine farms and slaughter plants. The selected strains were from the conventional and ABF production systems representative of the processing stages at farm and slaughter and the resistance patterns observed during the entire study. Briefly, a total of 21 groups of pigs that belong to two distinct production systems (11 conventional and 10 ABF farms) were sampled at the nursery farms (6 weeks of age) and finishing farms (within 48 hours of slaughter) followed by carcass sampling at the slaughter plant. Sample collection at the slaughter plant was done at three stages including pre-evisceration (before viscera is taken out), post-evisceration (after viscera is taken out) and post-chill. Prior to testing, the isolates were recovered from storage at -80^{o}C and streaked on Mueller-Hinton agar supplemented with 5% sheep blood. All the incubations were done under microaerobic conditions at 42 ^{o}C for 48 hours. Antimicrobial susceptibility testing was done using the agar dilution method as described below. Genotyping of the *C. coli* isolates was done by MLST and PFGE as described later in this section.

3.2.2 Antimicrobial susceptibility testing

The agar dilution method was used as recommended by the National Clinical Committee for Laboratory Standards (NCCLS) subcommittee on Veterinary Antimicrobial Susceptibility Testing for determining the susceptibility of *Campylobacter* isolates to different antimicrobials (National Committee for Clinical Laboratory Standards, 1999). We tested the isolates for their susceptibility against a panel of six antimicrobials. The list of antimicrobial with their abbreviations and range of concentrations used is: Chloramphenicol (Ch; 0.25-128 mg/l), Ciprofloxacin (Cip; 0.008-4 mg/l), Erythromycin (Ery; 0.06-32 mg/l), Gentamicin (Gen; 0.06-32 mg/l), Nalidixic acid (Nal; 0.25-128 mg/l) and Tetracycline (Tet; 0.06-32 mg/l) (13). All the antimicrobials were procured from Sigma (Sigma, MO, USA) except Ciprofloxacin (Serologicals Proteins, Inc., IL, USA). The NCCLS breakpoint interpretative criteria for *Enterobacteriaceae* family were used for all the antimicrobials except Erythromycin as recommended by NCCLS as the interpretive standard breakpoint levels for the *Campylobacteriaceae* family are not yet available (Ge et al., 2002). For Erythromycin (8 mg/liter), the breakpoint level used by National Antimicrobial Resistance Monitoring System (NARMS) was adopted (FDA/USDA/CDC, 1999). *C. jejuni* ATCC 33560 was used as the quality control (QC) organism for this test (Ge et al., 2002). The minimum inhibitory concentration-50 (MIC_{50}) breakpoints used for each antimicrobial were: Chloramphenicol (32 mg/l), Ciprofloxacin (4 mg/l), Erythromycin (8 mg/l), Gentamicin (16 mg/l), Nalidixic acid (32 mg/l) and Tetracycline (16 mg/l).

Fresh growth of *C. coli* isolates on sheep blood agar plates was diluted in 3 ml of Mueller-Hinton broth using a colorimeter (bioMerieux, NC, USA) to a concentration of 0.5 McFarland standards ($1X10^8$ CFU/ml). Two fold serial dilutions of the antimicrobials

were made in sterile distilled water with the above mentioned dilution range. One ml of the diluted antimicrobial in 2 ml of sheep blood was added to 17 ml of Mueller-Hinton agar making it a total of 20 ml agar media with the desired concentration of the antimicrobial. The diluted cultures (approximately $1X10^4$ CFU/inoculum) were then plated onto the antimicrobial plates using a Cathra replicator with 1 mm diameter pins (Oxoid Inc., Ontario, Canada). The plates were then incubated for 48 hours at 42^oC under microaerobic conditions and the MIC was recorded for each antimicrobial. Multidrug resistance (MDR) isolates here are defined as isolates that are resistant to three or more antimicrobials.

3.2.3 Multilocus sequence typing (MLST)

Genomic DNA purification of the isolates for sequencing was done using the Qiagen DNA purification kit (Qiagen, Valencia, CA). MLST of the seven housekeeping genes (*aspA, glnA, gltA, glyA, pgm, tkt* and *uncA*) for *C. coli* was done following the method described previously (Dingle et al., 2005). The primer sequence for the housekeeping genes are shown in Table 3.1. Briefly, all the housekeeping genes were amplified using PCR and the products were run on agarose gel to confirm the correct amplicon size. Purification of the PCR products was done using the QiaAmp PCR purification kit following the manufacturer's instructions (Qiagen, Valencia, CA). Sequencing reactions using the forward and reverse primers in separate wells was done using 2 µl of the BigDye Ready Reaction mix (version 3.1; Applied Biosystems, Foster City, California), 0.5 µl of 1:15 diluted primer, 5.5 µl of molecular grade deionized water and 2 µl of the purified PCR product. Sequencing reaction was performed on the

automated 3700 ABI capillary sequencer (Applied Biosystems, Foster City, California) with running conditions of 30 cycles of 96 ^{o}C for 10 s, 50 ^{o}C for 5 s and 60 ^{o}C for 4 min. We used the ClustalW program for aligning the forward and reverse sequences (www.ebi.ac.uk/clustalw). The allelic profiles and the STs were then generated by blasting the correct sequence size on the MLST website from the *Campylobacter* database (www.mlst.net). Dendrogram for the MLST data was generated using the START software (Jolley et al., 2001).

3.2.4 Pulsed field gel electrophoresis (PFGE)

PFGE was done following the rapid protocol for *Campylobacter* (Ribot et al., 2001). Briefly, 400 ml overnight culture cells were lysed and intact genomic DNA digested in agarose- embedded plugs with *SmaI* restriction enzyme. The digested DNA was then separated by using a contour-clamped homogeneous electric field (CHEF)-DRIII (Bio-Rad Laboratories, Hercules, CA) with the following conditions: 0.5xTBE (Tris-Borate 15 EDTA), 1% Seakem Gold agarose (FMC BioProducts, Rockland, ME), 14°C, 6V/cm for 18 h with switch times ranging from 6.75 to 38.35 s with an included angle of 120^{o}. *Salmonella* enterica serovar Braenderup 17 'Universal Marker' (kindly provided by Dr. Leslie Wolf, NCSLPH, NC) was used as the reference marker. Gels were stained with ethidium bromide for 30 min., destained three times for 20 min. each with distilled water and photographed using Alpha imager (Alpha 20 Innotech Corporation, San Leandro, CA). Analysis of PFGE data was performed using Bionumerics software (Applied Maths, Kortrijik, Belgium) using "different bands" algorithm for clustering and the Ward algorithm of tree building approach with 0.47% optimization and 0.48%

position tolerance determined by using procedures recommended by the manufacturer. Visual inspection of the patterns was performed as a final step for analysis.

3.2.5 Data Analysis

Simpson's index of diversity was calculated to compare the discriminatory power of the two genotyping methods used in this study (Hunter and Gaston, 1988). The formula used to calculate the index of discrimination (DI) is:

$$DI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

DI= Index of discrimination

N=Total number of strains in the sample population

S= the total number of types described

n_j= the number of strains belonging to the jth type.

The DI value shows the probability that two unrelated strains sampled from the test population would be placed into different cluster types. This value ranges between 0.0 and 1.0. The higher the index, the more discriminatory the method is.

The index of association (I_A) was determined using the START program to assess the clonality of the population (Maynard-Smith et al., 1993). An absolute value of zero (I_A =0) indicates that the population is freely recombining and is not clonal. A value of 1.0 indicates the high genetic diversity of isolates.

3.3 RESULTS

3.3.1 Diversity of STs across the two production systems based on MLST

Assignment of the allele frequencies and STs was done using algorithms described on the *Campylobacter* MLST database (www.Campylobacter.mlst.net). A total of 65 STs were generated from sequence typing of 100 *C. coli* isolates with 47 STs occurring singly and ST 1413 being the most common seen in 7 isolates from the carcass of ABF pigs (Table 3.2 and 3.3). Based on the allelic profiles of the housekeeping genes, 50 new STs were assigned for the first time after submitting the information to the MLST database. Twenty four out of the 50 new STs originated from the ABF swine production units. The remaining 27 new STs were from *C. coli* isolates from the conventional production system. Within individual production systems, we observed STs that were found specific to the processing stages either at the farm or slaughter with thirty eight out of the 65 STs were found to be specific to the farm and another 22 were found only at the slaughter stage. The three most predominant STs occurring in the database included ST-1413 (7 isolates), ST-854 (6 isolates) and ST-1123 (5 isolates) representing 18% of the isolates. Multiple STs were generated for individual antimicrobial resistance patterns with the majority being specific either to the farm or the slaughter stage (Table 3.3). For instance, ST -1413 (n= 7) was observed only among the *C. coli* ciprofloxacin resistant strains (resistance pattern: Cip Ery Nal Tet) isolated from the conventional nursery pigs. However, two additional isolates from the same farm and sharing the same resistance pattern as above had different STs (ST-1096; cluster number 9 and ST-19; cluster number19).

Based on the MLST dendrogram generated by the START program, we observed a total of 27 clusters with cluster number 9 being the largest (n= 18) (Figure 3.1). Seven isolates were represented by single branches and occurred independently without being a part of any group. Majority of the isolates (n=16) in cluster number 9 were from the nursery and finishing farms and included eight out of the 15 ciprofloxacin resistant *C. coli* isolates. We detected clusters that were specific to the production system or the processing stage. For example, clusters 2, 5, 6, 19 and 21 comprised of isolates from the farm while clusters 7, 8 and 24 included slaughter isolates only. Multiple STs were found among isolates from a single farm at different stages of processing both at farm and slaughter. For example, isolate IDs 2003 and 2032 with tetracycline resistance pattern (Tet) were isolated from the same ABF farm but were associated with ST-890 and 825 respectively. Similarly isolates number 3490 and 3491 were from the same pig (Tet resistant) reared in the conventional system but were associated with two different STs, ST-1130 and ST-854 respectively.

The I_A for the sample population was 0.293 indicating a weak clonal structure. However, the I_A values for the ABF and the conventional system were 0.279 and 0.535 respectively indicating a higher clonality of *C. coli* isolates from the conventional system. Simpson's index of diversity calculated for the MLST method was found to be 0.936. Such a high DI value shows that MLST has a very high discriminatory power.

3.3.2 Diversity among the 100 *C. coli* isolates based on PFGE fingerprinting

The *SmaI* digested genome of *C. coli* resulted in the generation on average 6-10 bands. Using 70% genetic similarity as the cut off, a total of 11 clusters were observed

with cluster 1 (17 isolates), 11 (16 isolates) and 3 (15 isolates) being the predominant ones comprising 48% of the total isolates (Figure 3.2). We followed the recommended criteria for interpreting PFGE banding patterns with isolates differing by one to three bands being most likely clonal (Tenover et al., 1995).

Three clusters had isolates grouped together based on their antimicrobial resistance pattern. These included cluster number 4 (n=4; Tet), cluster number 6 (n=3; Tet) and cluster number10 (n=6; Cip Ery Nal Tet). Isolates from cluster number 6 and number 10 were epidemiologically related being isolated from the same slaughter and farm groups. One group of isolate each in cluster one (isolate IDs 762, 3995, 4037) and 11 (isolate IDs 26, 793, 794) were epidemiologically related representing the nursery, finishing and the slaughter stages of two different ABF farms. However, the overall population exhibited considerable genotypic diversity. The remaining clusters were very diverse based on the location and time of isolation and the resistance patterns of these isolates. This indicates the diverse nature of the *C. coli* isolates. The PFGE method had a discriminatory index of 0.889.

3.3.3 Comparison of clustering by MLST and PFGE

We identified a total of 27 and 11 clusters for the 100 *C. coli* isolate genotyped using MLST and PFGE, respectively. The association of MLST STs with PFGE clusters is shown in Table 3.4. Out of the 65 MLST STs observed for the complete dataset, 53 STs (81.5%) representing 62 *C. coli* isolates were represented by a single PFGE cluster indicating that these isolates were not distinguished by the PFGE method. The remaining 12 STs were clustered in 2 to 3 PFGE clusters. A single PFGE cluster (cluster 4) was

found to be associated with a single ST (ST-854). The remaining 10 PFGE clusters were associated with multiple STs. PFGE type 1 was the most heterogeneous cluster and included 16 STs representing the farm and slaughter stages of both the production systems.

Each of the methods was able to further differentiate the isolates clustered together in a single group by either MLST or PFGE. For example, PFGE cluster type 1 (n = 17 isolates) and 11 (n = 16 isolates) were differentiated into 10 and 8 MLST clusters respectively. Similarly, MLST cluster 9 (n = 18 isolates) was represented by six PFGE clusters when typed by PFGE. However, we found that MLST had a better discriminatory power of 0.936 when compared to 0.889 for PFGE. Visual interpretation of the two dendrogram also reflected the ability of MLST to discriminate between isolates that were clustered in the same group by PFGE.

Overall, MLST was able to discriminate better between the *C. coli* isolates from the conventional and the ABF system. MLST had a higher throughputness as reactions can be carried out in 96 well plates. However, PFGE was more cost effective costing approximately $7.00 per reaction compared to $42 for MLST.

3.4 DISCUSSION

There are a multitude of studies that have reported the weak clonal population structure and the hypervariable genome of *Campylobacter* (Dingle et al., 2001; Dingle et al., 2005; On, 1998; Wassenaar et al., 1998). This makes the choice of using a genotyping method for determining the source of an outbreak or comparing isolates from different sources more complex and difficult to interpret. To solve this problem, an MLST scheme

was developed for *C. jejuni* and has been shown to be a reliable method for typing human, animal and environmental strains of this pathogen (Dingle et al., 2001; Colles et al., 2003; Manning et al., 2003; Sails et al., 2003a; Leatherbarrow et al., 2004). Recently, the MLST scheme was extended for typing *C. coli*, another important species of *Campylobacter* besides *C. jejuni* causing foodborne gastroenteritis (Dingle et al., 2005). Another technique used routinely for typing both *C. jejuni* and *C. coli* is PFGE (Newell et al., 2000; Fitzgerald et al., 2001; Cloak and Fratamico, 2002; Sails et al., 2003; Chu et al., 2004). This method is being used by PulseNet within the US for the nationwide surveillance of this pathogen along with other foodborne pathogens like *salmonella* and *Shigella* (Swaminathan et al., 2001). It has been used both for typing this pathogen and for discriminating between *C. jejuni* isolates responsible for 12 outbreaks in the US (Sails et al., 2003). These two methods have not been compared to investigate the genetic diversity of *C. coli* strains isolated from swine. The utility of both the methods as tools for understanding the epidemiology of this pathogen in the swine production environment is important. Therefore, in this study, we compared these two methods for genotyping 100 phenotypically diverse *C. coli* isolated from swine reared under conventional and antimicrobial free production systems and compared their discriminatory power, throughputness and group associations.

MLST and PFGE differentiated the isolates into 27 and 11 clusters respectively, exhibiting high level of discrimination depending on the farm type and the antimicrobial resistance pattern. Our results were consistent with other studies where both these methods have been shown to differentiate between closely related strains of *Campylobacter* (Sails et al., 2003; Sails et al., 2003a; Leatherbarrow et al., 2004). The

distribution of specific STs among the isolates at different stages of production and with particular resistance patterns indicated that certain STs were adapted to specific stage or production. Specific STs were found either at the farm or at the slaughter stage (Table 3.2 and 3.3). For example, ST-1413 was seen in *C. coli* isolates with Cip Ery Nal Tet resistance pattern and only in isolates from pigs at nursery farm in the conventional production system. Similar results have been reported by other studies where specific clones of *C. jejuni* have been found associated with particular niches (Colles et al., 2003). A study done in UK reported a *C. coli* strain that may have become adapted to persist in water and act as a source of infection to humans (Leatherbarrow et al., 2004).

The results observed in our study of specific STs being associated with specific production or processing stage such as slaughter may imply that not all strains detected at slaughter originated from the farm and other factors such as cross contamination during trucking and in holding pens remain a concern. The genotyping results provided evidence of multiple *Campylobacter* genotypes grouped together in different clusters. We observed clusters with isolates that were not related either temporally or spatially indicating significant genotypic diversity. Hume et al. reported the absence of shared genotype from isolates that were isolated from the sow, its respective piglets and the littermates highlighting the diverse genome of this pathogen (Hume et al., 2002). Similar observations were made when we analyzed the clusters with respect to the resistance patterns. Except a few STs that were restricted to specific resistance patterns (ST-1413 was associated with isolates with Cip Ery Nal Tet resistance pattern); most of the STs were found to be associated with multiple resistance patterns. *C. jejuni* isolates with

similar PFGE patterns but with different resistance patterns has been reported before (Chu et al., 2004).

We found MLST to have a slightly higher discriminatory power and throughput than PFGE. MLST was able to further discriminate between the clusters defined by PFGE. For example, two groups of isolates representing the nursery, finishing and slaughter plant levels for the same farm, that is, related temporally and spatially and placed in the same cluster by PFGE, were further discriminated by MLST. Many studies have reported the better discriminatory power of PFGE compared to MLST when used for typing *C. jejuni* (Sails et al., 2003; Sails et al., 2003a), but have not been compared for genotyping *C. coli*. MLST has been found to be as discriminatory as PFGE for distinguishing between temporally related isolates and the epidemic causing isolates in different outbreaks caused by *C. jejuni* (Sails et al., 2003). The ability of MLST to have the same value for epidemiological typing as that of PFGE, AFLP and ribotyping has also been demonstrated before (Duim et al., 2003). PFGE on the other hand gave us good discrimination but not as well as MLST. In some instances, a single cluster of MLST was also represented by multiple clusters of PFGE. These findings imply that both genotyping methods are very good and the use of both methods would be ideally a preferred way of genotyping. It should also be emphasized that the outcome of PFGE typing is susceptible to changes in the chromosome and inter and intragenomic recombinations making this method unsuitable for studying the long term global epidemiology of this pathogen (Hanninen et al., 1999; On, 1998; Wassenaar et al., 1998; Weijtens et al., 1999).

Analysis of clusters generated by the two methods revealed the high level of genotypic diversity present in *Campylobacter*. We detected clustering of isolates based

on the processing stage or sample type rather than the production system. Leatherbarrow et al. however reported no clustering seen in *C. coli* based on the sample type (Leatherbarrow et al., 2004). Seventeen clonal complexes have been defined so far for *C. jejuni* and association of these clonal complexes with the given host has been shown by MLST and PFGE (Colles et al., 2003; Dingle et al., 2001). Defining a clonal complex of *C. coli* in swine will help us in understanding its epidemiology in this host in the environment.

The overall *C. coli* population had a weaker clonal structure (I_A = 0.293) when compared to the I_A value of 0.57 for *C. jejuni* (Dingle et al., 2001). Our findings show that the *C. coli* population has low clonal structure and widespread genotypic diversity was seen. In *C. jejuni*, seventeen clonal complexes have been defined so far and association of these clonal complexes with the given host has been shown by MLST and PFGE (Colles et al., 2003; Dingle et al., 2001). Miller et al. recently defined two clonal complexes for *C. coli* (Millet et al., 2005). We found the *C. coli* population isolated from the conventional production system to be slightly more clonal than that of the ABF system. This could be attributed to the presence of different lineage of this species circulating in these production systems. Our results differ from other studies that have reported *C. coli* to be less diverse than *C. jejuni* by using MLST and AFLP (Dingle et al., 2005; Duim et al., 1999). However, it should also be noted that the isolates in the current study are not representative of the *C. coli* population existing in these systems and we are cautious that no generalization is deduced from the study.

3.5 Conclusions

This study highlights the high genotypic diversity of antimicrobial resistant *C. coli* in the swine production systems. Evidence of clonality in the conventional production system shows the ability of *C. coli* to adapt to specific niche. Sequence Type determination of the isolates has tremendous advantage for facilitated inter-laboratory comparisons. We emphasize that MLST has the potential to be used for studying the molecular epidemiology of *Campylobacter* due to its high discriminatory power, the simplicity of data handling and analysis, reproducibility of sequence data, high throughputness and ease with which data can be exchanged between different laboratories via the internet. The nucleotide sequence information generated for the housekeeping genes from different isolates can be used for phylogenetic analysis to better understand the population biology of this important pathogen.

3.6 Acknowledgements

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Table 3.1 Primer sequences for the seven housekeeping genes used to perform *Campylobacter coli* MLST

Locus	Forward	Reverse
aspA	Aspcoli S1 5'-CAACTTCAAGATGCAGTACC-3'	Aspcoli S2 5'-ATCTGCTAAAGTATGCATTGC-3'
glnA	Glncoli S1 5'-TTCATGGATGGCAAACCTATTG-3'	Glncoli S2 5'-GCTTTGGCATAAAAGTTGCAG-3'
gltA	Gltcoli S1 5'-GATGTAGTGCATCTTTTACTC-3'	Gltcoli S2 5'-AAGCGCTCCAATACCTGCTG-3'
glyA	Glycoli S1 5'-TCAAGGCGTTTATGCTGCAC-3'	Glycoli S2 5'-CCATCACTTACAAGCTTATAC-3'
pgm	Pgmcoli S1 5'-TTATAAGGTAGCTCCGACTG-3'	Pgmcoli S2 5'-GTTCCGAATAGCGAAATAACAC-3'
tkt	Tktcoli S1 5'-AGGCTTGTGTTTTCAGGCGG-3'	Tktcoli S2 5'-TGACTTCCTTCAAGCTCTCC-3'
uncA	Unccoli S1 5'-AAGCACAGTGGCTCAAGTTG-3'	Unccoli S2 5'-CTACTTGCCTCATCCAATCAC-3'

Table 3.2 Total number of *Campylobacter coli* isolates under each production system including the MLST STs and PFGE clusters

Production Type	Processing Stage	No. of Strains	No. of STs (%Diversity) [e]	No. of New STs (%) [b]	No. of Unique STs (%) [c]	PFGE Cluster (n) [d]
ABF [a] (Isolates tested =50)	Nursery	9	8 (89)	4 (50)	4 (50)	1, 3, 4, 5, 10, 11, (n=6)
	Finishing	27	20 (74)	11 (55)	11 (55)	1, 2, 3, 4, 5, 7, 8, 9, 11, (n=9)
	Pre-Evisceration	5	5 (100)	5 (100)	3 (60)	1, 8, 9, (n=3)
	Post-Evisceration	7	6 (86)	3 (50)	3 (50)	1, 5, 8, (n=3)
	Post-Chill	2	2 (100)	1 (50)	1 (50)	8, (n=1)
Conventional (Isolates tested =50)	Nursery	14	8 (57)	7 (88)	6 (75)	1, 5, 8, 9, 10, (n=5)
	Finishing	21	18 (86)	12 (67)	13 (72)	1, 2, 3, 4, 9, 11, (n=6)
	Pre-Evisceration	5	5 (100)	4 (80)	4 (80)	2, 3, (n=2)
	Post-Evisceration	9	9 (100)	2 (22)	7 (78)	3, 6, 8, 9, (n=4)
	Post-Chill	1	1 (100)	1 (100)	1 (100)	2, (n=1)

[a] Antimicrobial Free Farms; [b] Indicates Sequence Types that have been reported for the first time.

[c] Indicates Sequence Types that were found only in the specific processing stage.

[d] n: Indicates total PFGE clusters and the Cluster numbers where the Sequence Types were grouped.

[e] Indicates the percent diversity of STs observed in the total isolates tested for that group.

Table 3.3 MLST and PFGE data for the 100 *Campylobacter coli* isolates

Production	Processing	Resistance	Isolate	Housekeeping Genes used for MLST							ST	MLST	PFGE
System	Stage	Pattern	ID	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		Cluster	Cluster
ABF	Nursery	Nal	53	33	39	30	78	104	43	17	1068	4	5
ABF	Nursery	Pansusceptible	183	33	39	30	82	104	43	68	1112	22	11
ABF	Nursery	Pansusceptible	752	33	38	30	82	104	173	68	1134	18	11
ABF	Nursery	Ery Tet	26	33	39	37	82	104	43	68	1414	21	11
ABF	Nursery	Pansusceptible	252	33	39	37	82	104	43	68	1414	21	3
ABF	Nursery	Tet	762	33	39	30	174	118	35	17	1431	6	1
ABF	Nursery	Ery Nal Tet	7	33	39	46	82	104	43	68	1437	21	1
ABF	Nursery	Cip Nal Tet	260	33	153	30	82	104	44	17	1438	15	10
ABF	Nursery	Tet	206	33	38	30	82	104	43	17	854	4	4
ABF	Finishing	Tet	4010	33	38	30	82	104	43	17	854	4	4
ABF	Finishing	Tet	4082	33	38	30	82	104	43	17	854	4	4
ABF	Finishing	Tet	2003	33	38	30	82	104	35	36	890	9	3
ABF	Finishing	Ery Nal Tet	2112	33	38	30	82	104	35	17	1096	9	3
ABF	Finishing	Ery	793	33	38	30	82	152	173	68	1102	18	11
ABF	Finishing	Ery Tet	786	33	39	30	82	104	43	68	1112	22	2
ABF	Finishing	Pansusceptible	3518	53	38	44	82	118	35	36	1123	13	2
ABF	Finishing	Pansusceptible	3978	53	38	44	82	118	35	36	1123	13	8
ABF	Finishing	Nal Tet	953	33	38	30	82	104	173	68	1134	18	11
ABF	Finishing	Pansusceptible	2059	33	38	30	82	104	173	68	1134	18	11
ABF	Finishing	Pansusceptible	3819	33	38	30	82	104	173	68	1134	18	11
ABF	Finishing	Tet	4115	53	38	30	82	118	35	36	1200	13	8
ABF	Finishing	Cip Nal Tet	4076	33	39	132	82	104	44	17	1424	3	7
ABF	Finishing	Pansusceptible	3969	53	38	30	81	118	85	36	1426	1	11
ABF	Finishing	Ery Tet	3995	33	39	30	79	104	56	17	1427	5	1
ABF	Finishing	Ery	1987	33	39	30	82	118	35	36	1432	12	5
ABF	Finishing	Ery Nal Tet	952	33	39	134	82	104	56	17	1433	5	1
ABF	Finishing	Tet	4077	33	39	44	82	104	44	17	1436	3	7
ABF	Finishing	Ery Tet	1010	33	38	30	82	118	35	17	1446	9	3
ABF	Finishing	Ery Tet	4017	33	38	30	82	118	35	17	1446	9	3
ABF	Finishing	Pansusceptible	1038	33	38	32	82	104	35	68	1447	18	11

Table 3.3 continued

Production	Processing	Resistance	Isolate	Housekeeping Genes used for MLST							ST	MLST	PFGE
System	Stage	Pattern	ID	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		Cluster	Cluster
ABF	Finishing	Ery Tet	2164	53	39	44	82	118	35	17	1448	11	8
ABF	Finishing	Nal	3552	53	39	44	82	104	35	36	1450	10	8
ABF	Finishing	Cip Nal Tet	4075	33	39	46	82	104	44	17	1452	3	7
ABF	Finishing	Pansusceptible	4100	33	39	46	82	104	44	17	1452	3	7
ABF	Finishing	Tet	2032	33	39	30	82	113	47	17	825	2	9
ABF	Pre-Evisc.	Ery	794	33	39	47	82	104	43	36	1415	22	11
ABF	Pre-Evisc.	Pansusceptible	4029	33	39	30	82	118	35	36	1432	12	8
ABF	Pre-Evisc.	Pansusceptible	843	33	39	30	82	104	85	17	1445	4	1
ABF	Pre-Evisc.	Pansusceptible	3580	53	39	44	82	104	35	36	1450	10	8
ABF	Pre-Evisc.	Pansusceptible	3600	53	39	37	82	104	85	17	1451	25	1
ABF	Post-Evisc.	Ery Tet	4037	33	38	30	82	104	35	36	890	9	1
ABF	Post-Evisc.	Tet	2205	33	38	30	78	104	35	17	1113	8	5
ABF	Post-Evisc.	Tet	4042	53	38	44	82	118	35	36	1123	13	8
ABF	Post-Evisc.	Tet	4049	53	38	44	82	118	35	36	1123	13	8
ABF	Post-Evisc.	Ery	817	33	153	30	82	118	43	17	1416	15	1
ABF	Post-Evisc.	Ery	4055	33	39	30	82	118	35	36	1432	12	8
ABF	Post-Evisc.	Tet	2207	33	38	37	78	104	35	17	1449	8	5
ABF	Post-Chill	Pansusceptible	3635	33	39	30	82	104	43	17	828	4	8
ABF	Post-Chill	Ery	3630	53	38	30	81	118	35	36	1469	1	8
Conv	Nursery	Cip Ery Nal Tet	475	33	38	30	82	104	35	17	1096	9	5
Conv	Nursery	Cip Ery Nal Tet	524	33	38	30	82	104	117	17	1413	9	10
Conv	Nursery	Cip Ery Nal Tet	525	33	38	30	82	104	117	17	1413	9	1
Conv	Nursery	Cip Ery Nal Tet	549	33	38	30	82	104	117	17	1413	9	9
Conv	Nursery	Cip Ery Nal Tet	552	33	38	30	82	104	117	17	1413	9	9
Conv	Nursery	Cip Ery Nal Tet	554	33	38	30	82	104	117	17	1413	9	10
Conv	Nursery	Cip Ery Nal Tet	555	33	38	30	82	104	117	17	1413	9	10
Conv	Nursery	Cip Ery Nal Tet	556	33	38	30	82	104	117	17	1413	9	10
Conv	Nursery	Ery	84	33	39	44	82	104	35	36	1417	10	8
Conv	Nursery	Ery Tet	503	33	39	46	174	104	35	17	1418	6	1
Conv	Nursery	Cip Ery Gen Nal Tet	548	33	38	46	82	104	117	17	1419	19	9

Table 3.3 continued

Production	Processing	Resistance	Isolate	Housekeeping Genes used for MLST							ST	MLST	PFGE
System	Stage	Pattern	ID	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		Cluster	Cluster
Conv	Nursery	Cip Ery Gen Nal Tet	461	33	39	46	82	113	35	17	1440	2	1
Conv	Nursery	Ery Gen Tet	533	33	39	30	82	113	117	17	1465	2	1
Conv	Finishing	Ery Tet	3441	33	38	30	82	104	43	17	854	4	2
Conv	Finishing	Tet	3736	33	38	30	82	104	43	17	854	4	4
Conv	Finishing	Ery Tet	4246	33	39	30	82	104	43	36	1056	22	2
Conv	Finishing	Ery Tet	827	33	153	30	82	104	35	17	1059	15	3
Conv	Finishing	Ch Ery Tet	4511	33	38	30	82	104	35	17	1096	9	3
Conv	Finishing	Ery	872	33	38	30	82	104	85	17	1177	9	3
Conv	Finishing	Ery Tet	4489	33	39	44	82	104	35	36	1417	10	2
Conv	Finishing	Tet	648	32	39	115	115	104	85	17	1420	26	1
Conv	Finishing	Ery Tet	423	33	38	46	82	104	173	17	1422	19	11
Conv	Finishing	Ery Tet	429	33	39	46	82	104	47	17	1423	3	1
Conv	Finishing	Ch Cip Ery Nal Tet	4516	33	39	134	174	104	43	68	1425	21	1
Conv	Finishing	Ch Cip Ery Nal Tet	4517	33	39	134	174	104	43	68	1425	21	1
Conv	Finishing	Ery Tet	841	33	39	44	82	189	35	36	1429	10	2
Conv	Finishing	Tet	4396	32	153	30	82	104	44	36	1430	16	2
Conv	Finishing	Tet	448	32	38	44	82	104	43	17	1434	23	2
Conv	Finishing	Ery Tet	4490	33	153	30	82	104	44	17	1438	15	9
Conv	Finishing	Ery Nal Tet	458	33	38	30	82	104	173	17	1439	9	11
Conv	Finishing	Ery Nal Tet	459	33	38	30	82	104	173	17	1439	9	11
Conv	Finishing	Ery Tet	623	33	38	132	82	104	85	17	1442	19	3
Conv	Finishing	Tet	653	33	153	30	173	217	44	68	1466	14	11
Conv	Finishing	Ery	3451	33	39	30	82	113	47	17	825	2	9
Conv	Pre-Evisc.	Ery Tet	3480	33	38	30	82	104	35	36	890	18	3
Conv	Pre-Evisc.	Ery	690	33	38	44	82	104	117	36	1421	20	2
Conv	Pre-Evisc.	Ery	694	33	39	134	161	118	43	17	1435	24	3
Conv	Pre-Evisc.	Ery	699	32	38	44	82	113	43	36	1443	23	2
Conv	Pre-Evisc.	Ery Tet	4324	32	38	30	167	104	35	17	1453	7	3
Conv	Post-Evisc.	Tet	3491	33	38	30	82	104	43	17	854	4	11
Conv	Post-Evisc.	Tet	4559	33	38	30	82	104	85	68	887	18	6

Table 3.3 continued

Production	Processing	Resistance	Isolate	Housekeeping Genes used for MLST							ST	MLST	PFGE
System	Stage	Pattern	ID	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		Cluster	Cluster
Conv	Post-Evisc.	Ery Tet	3507	53	38	30	81	104	44	36	1097	1	3
Conv	Post-Evisc.	Ery Tet	892	33	38	134	161	104	43	17	1125	24	3
Conv	Post-Evisc.	Tet	3490	33	38	30	82	104	43	68	1130	18	11
Conv	Post-Evisc.	Tet	4549	33	153	30	82	104	43	36	1142	22	6
Conv	Post-Evisc.	Ery Tet	4338	58	38	30	167	118	35	17	1454	7	3
Conv	Post-Evisc.	Tet	4560	33	38	37	82	104	85	68	1455	17	6
Conv	Post-Chill	Ery Tet	4445	53	38	30	81	118	43	36	1428	1	2

[a] Antimicrobial Free Farms.

[b] Conventional farms

[c] Sequence Types indicates the unique number assigned on the basis of the allelic profile

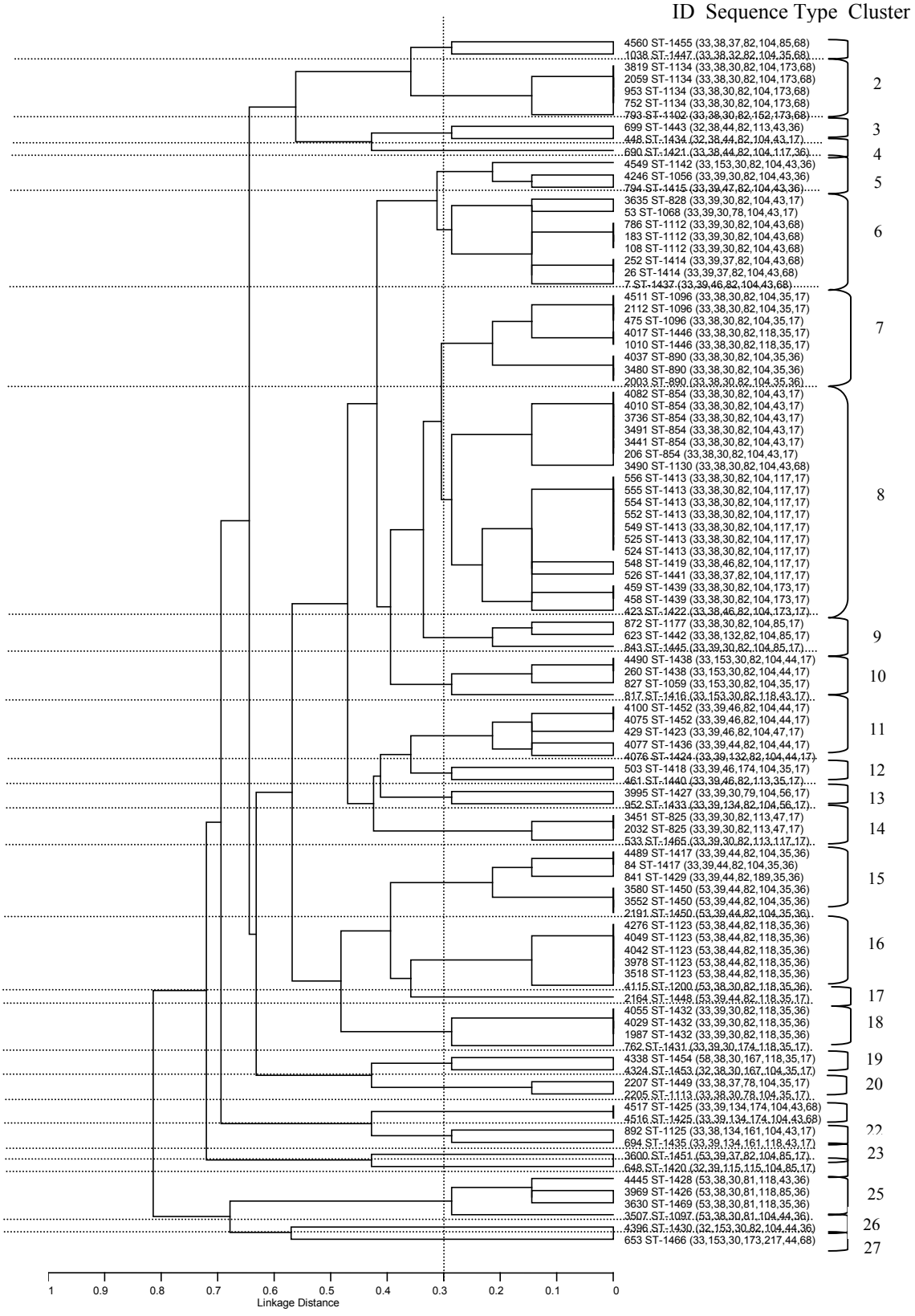
generated based on the allele nucleotide sequence number in the MLST database (www.mlst.net).

Table 3.4 MLST Sequence Type and PFGE cluster comparison for the 100 *Campylobacter coli* isolates

ST	PFGE Type										
	1	2	3	4	5	6	7	8	9	10	11
825									2		
828								1			
854		1		4							1
887						1					
890	1		2								
1056		1									
1059			1								
1068					1						
1096			2		1						
1097			1								
1102											1
1112		1									1
1113					1						
1123		1						4			
1125			1								
1130											1
1134											4
1142						1					
1177			1								
1200								1			
1413	1								2	4	
1414			1								1
1415											1
1416	1										
1417		1						1			
1418	1										
1419									1		
1420	1										
1421		1									
1422											1
1423	1										
1424							1				
1425	2										
1426											1
1427	1										
1428		1									
1429		1									
1430		1									
1431	1										
1432					1			2			
1433	1										
1434		1									
1435			1								
1436							1				
1437	1										
1438									1	1	
1439											2

Table 3.4 continued

1440	1										
1441										1	
1442			1								
1443		1									
1445	1										
1446			2								
1447											1
1448								1			
1449					1						
1450	1							2			
1451	1										
1452							2				
1453			1								
1454			1								
1455						1					
1465	1										
1466											1
1469								1			


ID Sequence Type Cluster
4560 ST-1455 (33,38,37,82,104,85,68)
1038 ST-1447 (33,38,32,82,104,35,68)
3819 ST-1134 (33,38,30,82,104,173,68)
2059 ST-1134 (33,38,30,82,104,173,68)
953 ST-1134 (33,38,30,82,104,173,68)
752 ST-1134 (33,38,30,82,104,173,68)
793 ST-1102 (33,38,30,82,152,173,68)
699 ST-1443 (32,38,44,82,113,43,36)
448 ST-1434 (32,38,44,82,104,43,17)
690 ST-1421 (33,38,44,82,104,117,36)
4549 ST-1142 (33,153,30,82,104,43,36)
4246 ST-1056 (33,39,30,82,104,43,36)
794 ST-1415 (33,39,47,82,104,43,36)
3635 ST-828 (33,39,30,82,104,43,17)
53 ST-1068 (33,39,30,78,104,43,17)
786 ST-1112 (33,39,30,82,104,43,68)
183 ST-1112 (33,39,30,82,104,43,68)
108 ST-1112 (33,39,30,82,104,43,68)
252 ST-1414 (33,39,37,82,104,43,68)
26 ST-1414 (33,39,37,82,104,43,68)
7 ST-1437 (33,39,46,82,104,43,68)
4511 ST-1096 (33,38,30,82,104,35,17)
2112 ST-1096 (33,38,30,82,104,35,17)
475 ST-1096 (33,38,30,82,104,35,17)
4017 ST-1446 (33,38,30,82,118,35,17)
1010 ST-1446 (33,38,30,82,118,35,17)
4037 ST-890 (33,38,30,82,104,35,36)
3480 ST-890 (33,38,30,82,104,35,36)
2003 ST-890 (33,38,30,82,104,35,36)
4082 ST-854 (33,38,30,82,104,43,17)
4010 ST-854 (33,38,30,82,104,43,17)
3736 ST-854 (33,38,30,82,104,43,17)
3491 ST-854 (33,38,30,82,104,43,17)
3441 ST-854 (33,38,30,82,104,43,17)
206 ST-854 (33,38,30,82,104,43,17)
3490 ST-1130 (33,38,30,82,104,43,68)
556 ST-1413 (33,38,30,82,104,117,17)
555 ST-1413 (33,38,30,82,104,117,17)
554 ST-1413 (33,38,30,82,104,117,17)
552 ST-1413 (33,38,30,82,104,117,17)
549 ST-1413 (33,38,30,82,104,117,17)
525 ST-1413 (33,38,30,82,104,117,17)
524 ST-1413 (33,38,30,82,104,117,17)
548 ST-1419 (33,38,46,82,104,117,17)
526 ST-1441 (33,38,37,82,104,117,17)
459 ST-1439 (33,38,30,82,104,173,17)
458 ST-1439 (33,38,30,82,104,173,17)
423 ST-1422 (33,38,46,82,104,173,17)
872 ST-1177 (33,38,30,82,104,85,17)
623 ST-1442 (33,38,132,82,104,85,17)
843 ST-1445 (33,39,30,82,104,85,17)
4490 ST-1438 (33,153,30,82,104,44,17)
260 ST-1438 (33,153,30,82,104,44,17)
827 ST-1059 (33,153,30,82,104,35,17)
817 ST-1416 (33,153,30,82,118,43,17)
4100 ST-1452 (33,39,46,82,104,44,17)
4075 ST-1452 (33,39,46,82,104,44,17)
429 ST-1423 (33,39,46,82,104,47,17)
4077 ST-1436 (33,39,44,82,104,44,17)
4076 ST-1424 (33,39,132,82,104,44,17)
503 ST-1418 (33,39,46,174,104,35,17)
461 ST-1440 (33,39,46,82,113,35,17)
3995 ST-1427 (33,39,30,79,104,56,17)
952 ST-1433 (33,39,134,82,104,56,17)
3451 ST-825 (33,39,30,82,113,47,17)
2032 ST-825 (33,39,30,82,113,47,17)
533 ST-1465 (33,39,30,82,113,117,17)
4489 ST-1417 (33,39,44,82,104,35,36)
84 ST-1417 (33,39,44,82,104,35,36)
841 ST-1429 (33,39,44,82,189,35,36)
3580 ST-1450 (53,39,44,82,104,35,36)
3552 ST-1450 (53,39,44,82,104,35,36)
2191 ST-1450 (53,39,44,82,104,35,36)
4276 ST-1123 (53,38,44,82,118,35,36)
4049 ST-1123 (53,38,44,82,118,35,36)
4042 ST-1123 (53,38,44,82,118,35,36)
3978 ST-1123 (53,38,44,82,118,35,36)
3518 ST-1123 (53,38,44,82,118,35,36)
4115 ST-1200 (53,38,30,82,118,35,36)
2164 ST-1448 (53,39,44,82,118,35,17)
4055 ST-1432 (33,39,30,82,118,35,36)
4029 ST-1432 (33,39,30,82,118,35,36)
1987 ST-1432 (33,39,30,82,118,35,36)
762 ST-1431 (33,39,30,174,118,35,17)
4338 ST-1454 (58,38,30,167,118,35,17)
4324 ST-1453 (32,38,30,167,104,35,17)
2207 ST-1449 (33,38,37,78,104,35,17)
2205 ST-1113 (33,38,30,78,104,35,17)
4517 ST-1425 (33,39,134,174,104,43,68)
4516 ST-1425 (33,39,134,174,104,43,68)
892 ST-1125 (33,38,134,161,104,43,17)
694 ST-1435 (33,39,134,161,118,43,17)
3600 ST-1451 (53,39,37,82,104,85,17)
648 ST-1420 (32,39,115,115,104,85,17)
4445 ST-1428 (53,38,30,81,118,43,36)
3969 ST-1426 (53,38,30,81,118,85,36)
3630 ST-1469 (53,38,30,81,118,35,36)
3507 ST-1097 (53,38,30,81,104,44,36)
4396 ST-1430 (32,153,30,82,104,44,36)
653 ST-1466 (33,153,30,173,217,44,68)
2
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5
6
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0.7
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0.2
0.1
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Linkage Distance

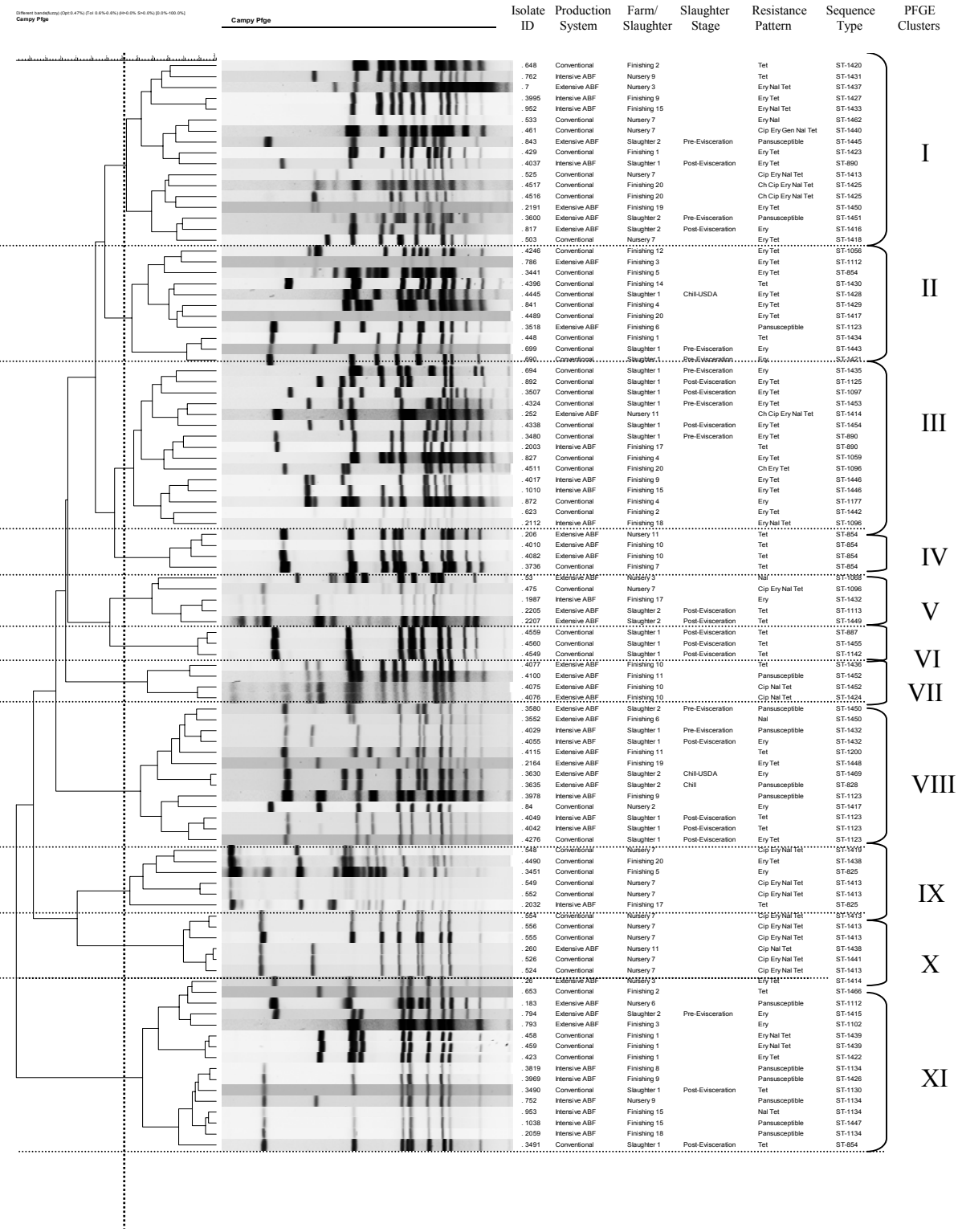

Figure 3.2 PFGE dendrogram for the 100 *Campylobacter coli* isolates showing the 11 clusters at 70% similarity between the band profiles

4. Genotypic diversity and Phylogenetic analysis of *Campylobacter coli* using Multi Locus Sequence Typing

Abstract

Multi locus sequence typing (MLST) has been routinely used for typing *Campylobacter jejuni* from diverse sources including humans, food animals and the environment. However, there is paucity of information on using this typing method for *C. coli* and other *Campylobacter* species to explain the molecular epidemiology of these species. In this study, a total of 151 *C. coli* isolates from swine reared in the conventional (n= 74) and the antimicrobial free (n = 77) production systems were typed by MLST. A total of 74 sequence types (STs) were identified with 51 STs being reported for the first time. Unique STs were found in each of the two production systems at farm and slaughter indicating niche adaptation. Phylogenetic analysis of the *C. coli* isolates showed evidence of dissemination of clonal population in both the production systems. The index of association for the two populations also corroborated the weak clonal structure of the pathogen with the two populations showing evidence of linkage equilibrium. Antimicrobial resistant isolates were represented by STs that were common in both the systems. The dissemination of the same *C. coli* population in the two production systems is concerning from food safety perspective. There was evidence of a clonal lineage present in *C. coli* isolates from the antimicrobial free system. The detection of such a lineage in *C. coli* will help us to compare isolates from diverse sources around the world and will be useful in understanding the global epidemiology of this pathogen.

4.1 Introduction

Epidemiological evidence has indicated that food animals including pigs, poultry and cattle act as reservoirs of *Campylobacter* strains that can infect humans (Sails et al., 2003; Manning et al., 2003; Hanninen et al., 2003; Fitzgerald et al., 2001). Studies done in Spain and UK have highlighted the importance of *C. coli* as an important human pathogen due to it ability to be more resistant to antimicrobials than *C. jejuni* and in causing more indigenously acquired foodborne diseases (Saenz et al., 2000; Tam et al., 2003). *C. coli* has been suggested to be particularly suited to the swine production environment and has been isolated from pigs on farm and at the slaughter level on the carcasses (Saenz et al., 2000; Nesbakken et al., 2003

Various Genotypic approaches have been used for this purpose including pulsed field gel electrophoresis (PFGE), *flaA* restriction fragment length polymorphism (*flaA*-RFLP), amplified length polymorphism (AFLP), ribotyping and recently multilocus sequence typing (MLST) (Lindmark et al., 2004; Fitzgerald et al., 2001; Duim et al., 1999, Manfreda et al., 2003; Dingle et al., 2001). *Campylobacter* genome has been shown to be hypervariable as shown by many studies and it is therefore important to use a typing method that accounts for the hypervariability and is based on indexing variation seen in the more conserved region of its genome (On et al., 1998; Weijtens et al., 1999; Harrington et al., 1997). One method that fits the above criteria is MLST, which has emerged as a suitable technique for epidemiological studies involving *Campylobacter.* This method is based on indexing variation in seven housekeeping genes and has been used routinely for *C. jejuni* typing and has recently been extended to *C. coli* (Dingle et al., 2001; Sails et al., 2003; Schouls et al., 2003; Dingle et al., 2005). The other advantage of this method is the amenability of the data to be used for phylogenetic analysis.

MLST data has been used for defining the clonal complexes in *C. jejuni* and a total of 17 different complexes have been reported (Dingle et al., 2005). Several of these complexes that are reported in humans have also been shown to exist in animals (Dingle et al., 2001; Manning et al., 2003; Colles et al., 2003). This scheme was extended to type *C. coli* isolates from humans and food animals as reported in a recent study conducted in UK (Dingle et al., 2005). The authors of that study compared the population biology of *C. coli* with *C. jejuni* and reported 95% similarity at the amino acid sequence level for the seven MLST loci. The focus of using MLST for characterizing *C. jejuni* is understandable considering it is the responsible for causing more than 90% of infections in humans. Consequently, the major focus is on *C. jejuni* and only two complexes have been recently been defined in *C. coli* (Miller et al., 2005). This study was done to characterize 151 *C. coli* isolates using MLST that were isolated from swine and swine carcasses from two distinct production systems, namely, the conventional and the antimicrobial free (ABF) systems. The main objective of this study was to determine the presence of clonal complexes of *C. coli*, compare the *C. coli* population in the two production systems, and determine their genetic diversity at farm and slaughter.

4.2 Materials and Methods

4.2.1 Bacterial isolates

C. coli isolates in this study (n = 151) were isolated from swine fecal samples and swine carcass swabs from farm and slaughter plant respectively. The isolates were selected from a collection of 1459 *C. coli* isolates that were isolated from a cross-sectional study conducted in swine reared under the conventional and antimicrobial free production (ABF)

system in North Carolina, United States (Chapter # 2). The 151 isolates comprised of 77 isolates from the ABF system (50 farm: 27 slaughter) and 74 from the conventional production system (46 farm; 28 slaughter).

4.2.2 DNA purification

Campylobacter isolates stored at -80 ^{o}C in brain heart infusion broth (Becton Dickinson, NJ, USA) with 35% DMSO (Sigma, MO, USA) were streaked on Mueller-Hinton agar supplemented with 5% sheep blood and incubated under microaerobic conditions at 42 ^{o}C for 48 hours. DNA isolation from the isolates was done using the Qiagen DNA purification kit (Qiagen, Valencia, CA). All the isolates were confirmed to be *C. coli* by PCR targeting the *ceuE* gene using the forward primer CC2- 5'-GATTTTATTATTTGTAGCAGCG-3' and the reverse primer CC3- 5'-TCCATGCCCTAAGACTTAACG-3' (Hani and Chan, 1995).

4.2.3 Antimicrobial susceptibility testing

The agar dilution method was used as recommended by the National Clinical Committee for Laboratory Standards (NCCLS) subcommittee on Veterinary Antimicrobial Susceptibility Testing for determining the susceptibility of *Campylobacter* isolates to different antimicrobials (NCCLS, 1999). We tested the isolates for their susceptibility against a panel of six antimicrobials. The list of antimicrobial with their abbreviations and range of concentrations used is: Chloramphenicol (Ch; 0.25-128 mg/l), Ciprofloxacin (Cip; 0.008-4 mg/l), Erythromycin (Ery; 0.06-32 mg/l), Gentamicin (Gen; 0.06-32 mg/l), Nalidixic acid (Nal; 0.25-128 mg/l) and Tetracycline (Tet; 0.06-32 mg/l) (Ge et al., 2003). All the

antimicrobials were procured from Sigma (Sigma, MO, USA) except ciprofloxacin (Serologicals Proteins, Inc., IL, USA). The NCCLS breakpoint interpretative criteria for *Enterobacteriaceae* family were used for all the antimicrobials except erythromycin as recommended by NCCLS as the interpretive standard breakpoint levels for the *Campylobacteriaceae* family are not yet available. For erythromycin (8 mg/liter), the breakpoint level used by National Antimicrobial Resistance Monitoring System (NARMS) was adopted (FDA/USDA/CDC, 1999). *C.jejuni* ATCC 33560 was used as the quality control (QC) organism for this test (FDA/USDA/CDC, 1999). The minimum inhibitory concentration-50 (MIC_{50}) breakpoints used for each antimicrobial were: Chloramphenicol (32 mg/l), Ciprofloxacin (4 mg/l), Erythromycin (8 mg/l), Gentamicin (16 mg/l), Nalidixic acid (32 mg/l) and Tetracycline (16 mg/l).

4.2.4 Multi Locus Sequence Typing (MLST)

MLST of the seven housekeeping genes (*aspA, glnA, gltA, glyA, pgm, tkt* and *uncA*) for *C. coli* was done following the method described previously (Dingle et al., 2005). Briefly, all the housekeeping genes were amplified using specific primers. The genes were amplified using PCR and the products were run on agarose gel to confirm the correct amplicon size. Purification of the PCR products was done using the QiaAmp PCR purification kit following the manufacturer's instructions (Qiagen, Valencia, CA). Sequencing reactions using the forward and reverse primers in separate wells was done using 2 µl of the BigDye Ready Reaction mix (version 3.1; Applied Biosystems, Foster City, California), 0.5 µl of 1:15 diluted primer, 5.5 µl of molecular grade deionized water and 2 µl of the purified PCR product. Sequencing reaction was performed on the automated 3700 ABI capillary sequencer

(Applied Biosystems, Foster City, California) with running conditions of 30 cycles of 96 ^{o}C for 10 s, 50 ^{o}C for 5 s and 60 ^{o}C for 4 min. We used the ClustalW program for aligning the forward and reverse sequences (www.ebi.ac.uk/clustalw). The allelic profiles and the sequence types (STs) were then generated by multiple sequence alignment of the correct sequence size on the MLST website from the *Campylobacter* database (www.mlst.net).

4.2.5 Data Analysis

The ST most likely to be the founder of the clonal complex was determined using the eBURST software (Feil et al., 2004). We used the stringent group definition of six or more shared alleles at each locus unlike four in similar studies done before. Isolates fulfilling this definition were considered as members of the same clonal complex. We used the bootstrapping method (re-sampling 1000 times) to provide statistical confidence to assignment of the ST as the putative founder of the complex. Degree of clonality within each data set was defined by determining the index of association (I_A) using the START program (Jolley et al., 2001). An absolute value of zero (I_A=0) indicates a freely recombining population that is not clonal. An I_A value of 1.0 indicates the high genotypic diversity of isolates. Phylogenetic analysis of the isolates was done using the MEGA software (Kumar et al., 2003). Other analysis including the ratio of nonsynonymous to synonymous substitutions (d_N / d_S), fixed differences, shared polymorphisms and estimate of gene flow analysis (F_{ST}) was determined using the DnaSP software version 4.00 (Rozas, J. et al., 2003). The association of STs with the production systems was carried out using the χ^2 test (Minitab Inc. PA, USA) and Fisher's exact two-tailed test wherever applicable. A value of $P \leq 0.05$ was considered statistically significant.

4.3 Results

4.3.1 MLST allele sequences diversity across the two production systems

Alleles for the seven loci were generated for all the 151 *C. coli* isolates from the two production systems (Table 4.1). After concatenating the sequences for all the seven alleles (3309 bp) for the 151 isolates, a total of 33 alleles with 2.08 % variable sites (n = 69) were observed. Within individual production systems, number of alleles from the ABF system varied from two for the *aspA* locus to nine for the *tkt* locus and from three (*aspA*, *uncA*) to eight for the *glyA* locus for the conventional system (Table 4.3). Percent variable sites for the alleles in isolates from ABF and the conventional system varied from 0.4% (*glnA*, *uncA*) to 12.4% to 11.7% for the *gltA* locus respectively. The d_N/d_S ratio (non-synonymous to synonymous base substitution) was less than one for all the housekeeping genes and varied from 0 to 0.15 in the isolates from ABF system and from 0 to 0.66 in the conventional system. The d_N/d_S ratio of less than one indicates stability of the sequence and against amino acid substitution.

4.3.2 Sequence type diversity across the two production systems

For the 151 *C. coli* isolates typed by MLST, we observed a total of 74 unique STs among which 51 STs were never reported before on the MLST database (Table 4.1 and 4.2). Twenty six STs occurred in more than one instance. Overall, a total of 51 STs from the ABF system (31 STs from farm and 20 STs from slaughter) and another 49 STs from the conventional production system (30 STs from farm and 19 STs from slaughter) were identified. Although the major focus of this study was to determine the genetic diversity of *C. coli* at the production level at farm and slaughter, the distribution of individual STs within the

processing stages at farm (nursery and finishing stages) and at slaughter (pre-evisceration, post-evisceration and post-chill) are shown in Table 4.2.

We observed STs that were unique to both the production system on farm and at slaughter. The predominant STs observed among the ABF *C. coli* isolates included ST-1112 (n = 6) and ST-1123 (n = 5) while ST-1413 (n = 9) and ST-1096 (n = 6) were the most frequent STs observed in the isolates from the conventional system. The unique STs observed in the two production systems are shown in Table 4.4. For example, ST-1413 was confined to isolates from the farm under the conventional system only. Overall, 26 STs were found to be unique among isolates from two production systems. Only ST- 1112 was significantly associated with the ABF production system ($P = 0.05$). ST-1123 and ST-1096 were not significantly associated with the ABF and the conventional systems ($P = 0.11$ and 0.31, respectively). Unique STs were observed at farm or slaughter.

4.3.3 Distribution of ST among the antimicrobial resistant isolates

Individual resistance patterns were represented by multiple STs across both the systems. For instance, ST-1413 (n = 7) was observed only among the *C. coli* ciprofloxacin resistant strains (resistance pattern: Cip Ery Nal Tet) isolated from the conventional nursery pigs. However, two additional isolates from the same farm and sharing the same resistance pattern as above had different STs (ST-1096 and ST-19). Similarly, resistance pattern Ery Nal Tet was represented by STs 1096, 1437, 1433 in the ABF system and ST-1439 in the conventional system implying strains with this resistance pattern are not necessarily clonal.

4.3.4 *C. coli* genotype comparison across and within the two production systems

A low F_{ST} value of 0.02, which is close to the minimum value of zero, indicated high level of gene flow between the 77 and 74 *C. coli* isolates from the ABF and the conventional production systems (Table 4.5). The two populations of *C. coli* had no fixed differences between them and had 67 shared mutations. Radial neighbor-joining tree constructed using the concatenated gene sequences for the unique STs (3309 bp) further corroborated the above result showing clustering of the isolates irrespective of the production system or the processing stage (Figure 4.1). As shown in Figure 4.2, isolates from the farm and slaughter stages were clustered together and no clear separation was seen. In case of isolates from the conventional system (Figure 4.3), we observed clustering of isolates from the farm and slaughter in separate groups.

4.3.5 *C. coli* clonal complex determination

The eBURST software was used to generate a clonal complex using the 74 STs generated for the 151 *C. coli* isolates. ST-828 was predicted as the putative founder of the clonal complex with 121 out of the total 151 *C. coli* isolates as members of this complex (Figure 4.2). However, the bootstrap values of 54 % for this group did not provide the level of confidence for ST-828 (isolate from ABF system) to be the putative founder of the group (Table 4.6). Reassigning the data separately for the two production systems, we observed 57 out of the 77 isolates from the ABF system grouped under one lineage with a high bootstrap value of 73 % (Figure 4.3; Table 4.7). The lineage created for isolates from the conventional system a poor bootstrap value of 53 % (Figure 4.4; Table 4.8). The I_A for the whole population was 0.37 and varied from 0.34 for isolates from the ABF system to 0.62 for the conventional system.

4.4 Discussion

C. coli is an important bacterial pathogen and the ranks next to *C. jejuni* in causing campylobacteriosis in humans. A recent study conducted in UK has highlighted the importance of *C. coli* as a human pathogen causing over 25,000 cases of acquired foodborne disease (Tam et al., 2003). Various studies have reported the high prevalence of this species in pigs and environment (Saenz et al., 2000; Pezzotti et al., 2003; Padungton and Kaneene, 2003). To understand the population biology of *C. coli*, a MLST scheme was developed similar to that has been described for *C. jejuni* previously (Dingle et al., 2005). MLST has been successfully used for determining the genetic diversity of *C. jejuni* in humans, food animals, environment, in outbreak investigations and in understanding its phylogenetic makeup (Sails et al., 2003; Dingle et al., 2002, Colles et al., 2003). A total of 17 clonal complexes have been defined so far for *C. jejuni* and they have been very helpful to compare isolates from all over the world. Recently, a study by Millet et al., has reported two clonal complexes for *C. coli* consisting of isolates from diverse sources including humans, poultry pigs and sheep (Miller et al., 2005). In this study, we attempt to define clonal complex in *C. coli* isolated from swine reared in two distinct production systems at farm and slaughter. The detection of such a clonal complex will help us in better understanding the population biology and the epidemiology of this pathogen in humans and animals.

Data from our study deposited 51 new STs which can be accessed at the MLST website (www.mlst.net). At present, there are a total of 133 STs for *C. coli* isolated from pigs from various parts of the world. Unique STs were observed among the two production systems. This indicated that specific STs have adapted to these production systems. The special growth conditions and different environment of the conventional and ABF farms may

have applied selective pressure on specific STs to adapt to them. Further discrimination of unique STs was also observed at the farm and slaughter stages of processing. This further highlights the above statement of niche adaptation of STs. A study conducted in UK has reported the pigs to be colonized by specific STs indicating the host preference (Dingle et al., 2005). Similar studies conducted on *C. jejuni* have reported the association of STs complexes to specific niche (Manning et al., 2003; Dingle et al., 2002). Host adapted genotypes of *Campylobacter* species have been reported in humans, pigs, cattle, turkey, poultry and environmental sources (Dingle et al., 2002; Leatherbarrow et al., 2004; Lindmark et al., 2004, Schouls et al., 2003; Fitzgerald et al., 2001; Duim et al., 2003). However, besides the unique STs, other STs that were common to both the systems were observed at farm and slaughter. The results observed in our study of specific STs being associated with specific production or processing stage such as slaughter may imply that not all strains detected at slaughter originated from the farm and other factors such as cross contamination during trucking and in holding pens remain a concern.

Nucleotide diversity and percent variability was highest for the *gltA* locus across both the production systems. Although *C. coli* has been shown to have more diverse sequences than *C. jejuni* (Dingle et al., 2005), one potential reason for this high variability could the import of sequences from *C. jejuni* at this locus. High variability observed in previous studies in *C. jejuni* at the *uncA* locus with addition of allele 17 that originated in *C. coli* has been hypothesized (Dingle et al., 2001; Colles et al., 2003). Allele 30 of *gltA* gene was observed in this study in 70% of the isolates and represents a highly divergent allele sequence, which is responsible for the high nucleotide diversity observed at this locus. For example, ST-1415 had 47 nucleotide changes displaying extreme sequence divergence. The transfer of gene

fragment via interspecies recombination can explain such high divergence from *C. jejuni*. Similarly, horizontal transfer of *pgm* gene from *C. jejuni* to *C. coli* showing interspecies recombination has been also reported (Schouls et al., 2003). The highly divergent allele sequence due to recombinational event is another indication of a weak clonal population (Meinersmann et al., 2002).

Genetic diversity of the *C. coli* population was reflected by the low index of association values for both the overall as well as in the population existing at the production system level. The I_A for the whole population was low (0.37) and indicated a weak clonal structure of *C. coli* similar to that of *C. jejuni* (Dingle et al., 2001; Manning et al., 2003). The I_A for the *C. coli* population from the conventional system was high (0.62) and indicated a slightly more clonal structure to it. MLST of more isolates from these two systems will be done in the future and could shed more light on the reasons for the above observations. We observed same STs associated with isolates that were not related either temporally or spatially indicating significant genotypic diversity. Hume et al., reported the absence of shared genotype from isolates that were isolated from the sow, its respective piglets and the littermates highlighting the diverse genome of this pathogen (Hume et al., 2002). Similar observations were made when we analyzed the STs with respect to the resistance patterns. Barring a few STs that were restricted to specific resistance patterns (ST-1413 was associated with isolates with Cip Ery Nal Tet resistance pattern); most of the STs were found to be associated with multiple resistance patterns again highlighting the diverse genetic makeup of this species.

Radial neighbor-joining phylogenetic tree constructed using the unique STs observed in the two production systems did not show differentiation between the two populations.

Eventhough the I_A for the *C. coli* population from the conventional system was high (0.62), the unique STs originating at the farm and slaughter were grouped together with those from the ABF system showing lack of segregation. The evidence was further strengthened by the detection of 67-shared mutations and zero fixed differences between the two populations. The low F_{ST} value of 0.02 after concatenating the loci (3309 bp) and also for all the individual loci indicated that there was no difference between the two populations at the production level and in fact represents the same population with linkage equilibrium between the alleles. Previous studies in *C. jejuni* have reported its weak clonal structure based on the F_{ST} values and the I_A indicating the weak clonal structure for this species too (Colles et al., 2003, Dingle et al., 2001). This result has important implications as it indicates that irrespective of the different growing conditions of these two distinct production systems, the same population of *C. coli* is disseminated in them. This might explain the reason for the high prevalence of antimicrobial resistant *C. coli*, including ciprofloxacin resistant strains, in the ABF production system despite the fact that no antimicrobials are used either for therapeutic or growth promotion purpose.

The identification of 17 clonal complexes for *C. jejuni* has tremendously helped in comparing isolates from all over the world based on distribution of the particular isolate in any of these complexes (Dingle et al., 2002; Dingle et al., 2005 Manning et al., 2003; Sails et al., 2003). Dingle et al characterized 814 isolates of *C. jejuni* isolates from various sources including humans, animals, food and environment using MLST with 748 isolates found to be members of one of these complexes (Dingle et al., 2002). The authors demonstrated the ability of each clonal complex to be used as an epidemiological unit and help in characterizing *Campylobacter* isolates from diverse sources. However, no one has attempted

to define a clonal population for *C. coli* primarily because the focus in on *C. jejuni* being primarily responsible for humans campylobacteriosis. We observed evidence of an apparent lineage only in case of *C. coli* isolates from the ABF system (bootstrap value of 74 %) and not in case of either the conventional system (bootstrap value of 53 %) or for the overall population (bootstrap value of 54 %). A total of 44 out of the 77 isolates were placed in this group with ST-828 having the highest number of single locus variants (n = 8). These results were in contrast to the I_A values, which determined isolates from the conventional system to have a slightly more clonal structure compared to those from the ABF system. Analysis of the eBURST output was unable to provide confidence in defining a lineage in the conventional system. However, clustering of the isolates from farm and slaughter was observed in the image (Figure 4.4). We acknowledge that typing of more isolates in case of a weakly clonal population, especially including isolates from diverse sources; will help in better defining this apparent lineage.

One interesting observation was regarding the putative founder ST-828 found associated with a single *C. coli* isolate that was recovered from the carcass sample of an ABF reared pig. This ST has previously been reported in a *C. coli* isolate from human stool in UK who had recently traveled to Israel and was suffering with gastroenteritis (Dingle et al., 2005). Although, information on the food product implicated for causing the infection in this individual was not available, it does indicate the ability of this ST to cause infection in humans and exist in pigs as well. It is however difficult to link these two geographically distinct isolates together. The occurrence of the same *C. jejuni* ST-403 complex in isolates from humans at the Dutch Caribbean island of Curacao and from pig sampled in the UK and presence of related genotypes of *C. jejuni* in poultry and humans reiterates the above point

(Manning et al., 2003, Michaud et al., 2005). The *C. coli* isolate with ST-828 was isolated from the slaughter plant and its isolation from the carcass could be due to cross-contamination from humans or environmental sources like cutting instruments. Environmental contamination of the carcasses at the slaughter plant has been reported before where *Campylobacter* isolated from the workers gloves, gambrel table and the dehairer had the same genotype as *C. coli* and *C. jejuni* isolates from the swine carcasses indicating cross contamination (Cloak and Fratamico, 2002).

4.5 Conclusions

MLST of *C. coli* isolates from two distinct pig production systems highlighted the weak clonal population and the diverse genetic makeup of this species. The detection of unique STs at farm and slaughter showed niche adaptation. Linkage equilibrium between the isolates from the two production systems is an important observation and explains the high antimicrobial resistance exhibited irrespective of use of antimicrobials at the farm for growth promotion or therapeutic use. This observation has important implications as it indicates dissemination of *C. coli* isolates between the two production systems. Despite the genetic similarity of the isolates at the production level, there was evidence of lineage in the ABF system. Given the potential of pig isolates to cause disease in humans, we recommend testing of more *C. coli* isolates in pigs.

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Table 4.1 MLST of 151 *Campylobacter coli* isolates from conventional and ABF production systems with their sequence types and resistance patterns

Isolate ID	Isolation Source	Production System	Sequence Type	Allele Numbers							Resistance Pattern
				aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	
7	Pig Feces	ABF	1437	33	39	46	82	104	43	68	Ery Nal Tet
10	Pig Feces	ABF	1112	33	39	30	82	104	43	68	Nal Tet
26	Pig Feces	ABF	1414	33	39	37	82	104	43	68	Ery Tet
53	Pig Feces	ABF	1068	33	39	30	78	104	43	17	Nal
133	Pig Feces	ABF	1112	33	39	30	82	104	43	68	Nal Tet
142	Pig Feces	ABF	1416	33	153	30	82	118	43	17	Tet
183	Pig Feces	ABF	1112	33	39	30	82	104	43	68	Pansusceptible
206	Pig Feces	ABF	854	33	38	30	82	104	43	17	Tet
218	Pig Feces	ABF	1463	33	153	30	82	104	85	17	Nal Tet
252	Pig Feces	ABF	1414	33	39	37	82	104	43	68	Pansusceptible
260	Pig Feces	ABF	1438	33	153	30	82	104	44	17	Cip Nal Tet
271	Pig Feces	ABF	1450	53	39	44	82	104	35	36	Pansusceptible
743	Pig Feces	ABF	1130	33	38	30	82	104	43	68	Ery Tet
752	Pig Feces	ABF	1134	33	38	30	82	104	173	68	Pansusceptible
756	Pig Feces	ABF	1444	33	39	30	82	104	171	17	Ery
762	Pig Feces	ABF	1431	33	39	30	174	118	35	17	Tet
786	Pig Feces	ABF	1112	33	39	30	82	104	43	68	Ery Tet
793	Pig Feces	ABF	1102	33	38	30	82	152	173	68	Ery
948	Pig Feces	ABF	1177	33	38	30	82	104	85	17	Ery Nal
952	Pig Feces	ABF	1433	33	39	134	82	104	56	17	Ery Nal Tet
953	Pig Feces	ABF	1134	33	38	30	82	104	173	68	Nal Tet
988	Pig Feces	ABF	1432	33	39	30	82	118	35	36	Ery Nal
1010	Pig Feces	ABF	1446	33	38	30	82	118	35	17	Ery Tet
1038	Pig Feces	ABF	1447	33	38	32	82	104	35	68	Pansusceptible
1987	Pig Feces	ABF	1432	33	39	30	82	118	35	36	Ery
2003	Pig Feces	ABF	890	33	38	30	82	104	35	36	Tet
2032	Pig Feces	ABF	825	33	39	30	82	113	47	17	Tet
2059	Pig Feces	ABF	1134	33	38	30	82	104	173	68	Pansusceptible

Table 4.1 continued

2094	Pig Feces	ABF	1096	33	38	30	82	104	35	17	Ery
2112	Pig Feces	ABF	1096	33	38	30	82	104	35	17	Ery Nal Tet
2164	Pig Feces	ABF	1448	53	39	44	82	118	35	17	Ery Tet
2182	Pig Feces	ABF	1112	33	39	30	82	104	43	68	Ery
2191	Pig Feces	ABF	1450	53	39	44	82	104	35	36	Ery Tet
3518	Pig Feces	ABF	1123	53	38	44	82	118	35	36	Pansusceptible
3552	Pig Feces	ABF	1450	53	39	44	82	104	35	36	Nal
3804	Pig Feces	ABF	1055	33	39	30	82	104	47	17	Cip Ery Nal Tet
3813	Pig Feces	ABF	825	33	39	30	82	113	47	17	Ch Ery Tet
3814	Pig Feces	ABF	825	33	39	30	82	113	47	17	Ch Ery Tet
3819	Pig Feces	ABF	1134	33	38	30	82	104	173	68	Pansusceptible
3969	Pig Feces	ABF	1426	53	38	30	81	118	85	36	Pansusceptible
3978	Pig Feces	ABF	1123	53	38	44	82	118	35	36	Pansusceptible
3995	Pig Feces	ABF	1427	33	39	30	79	104	56	17	Ery Tet
4010	Pig Feces	ABF	854	33	38	30	82	104	43	17	Tet
4017	Pig Feces	ABF	1446	33	38	30	82	118	35	17	Ery Tet
4075	Pig Feces	ABF	1452	33	39	46	82	104	44	17	Cip Nal Tet
4076	Pig Feces	ABF	1424	33	39	132	82	104	44	17	Cip Nal Tet
4077	Pig Feces	ABF	1436	33	39	44	82	104	44	17	Tet
4082	Pig Feces	ABF	854	33	38	30	82	104	43	17	Tet
4100	Pig Feces	ABF	1452	33	39	46	82	104	44	17	Pansusceptible
4115	Pig Feces	ABF	1200	53	38	30	82	118	35	36	Tet
794	Carcass Swab	ABF	1415	33	39	47	82	104	43	36	Ery
798	Carcass Swab	ABF	1056	33	39	30	82	104	43	36	Ery
843	Carcass Swab	ABF	1445	33	39	30	82	104	85	17	Pansusceptible
849	Carcass Swab	ABF	1445	33	39	30	82	104	85	17	Ery Tet
2196	Carcass Swab	ABF	1467	33	38	30	167	104	43	17	Tet
2197	Carcass Swab	ABF	1467	33	38	30	167	104	43	17	Tet
3580	Carcass Swab	ABF	1450	53	39	44	82	104	35	36	Pansusceptible
3600	Carcass Swab	ABF	1451	53	39	37	82	104	85	17	Pansusceptible
4029	Carcass Swab	ABF	1432	33	39	30	82	118	35	36	Pansusceptible
4032	Carcass Swab	ABF	1470	53	38	30	82	104	35	36	Ery Tet
812	Carcass Swab	ABF	1112	33	39	30	82	104	43	68	Ery Tet
817	Carcass Swab	ABF	1416	33	153	30	82	118	43	17	Ery
2203	Carcass Swab	ABF	1096	33	38	30	82	104	35	17	Ch Ery Gen

Table 4.1 continued

2205	Carcass Swab	ABF	1113	33	38	30	78	104	35	17	Tet
2207	Carcass Swab	ABF	1449	33	38	37	78	104	35	17	Tet
3616	Carcass Swab	ABF	1468	53	38	44	82	118	117	36	Pansusceptible
4037	Carcass Swab	ABF	890	33	38	30	82	104	35	36	Ery Tet
4042	Carcass Swab	ABF	1123	53	38	44	82	118	35	36	Tet
4044	Carcass Swab	ABF	1123	53	38	44	82	118	35	36	Ch Ery Nal Tet
4049	Carcass Swab	ABF	1123	53	38	44	82	118	35	36	Tet
4055	Carcass Swab	ABF	1432	33	39	30	82	118	35	36	Ery
4089	Carcass Swab	ABF	1058	33	39	30	82	104	35	17	Pansusceptible
2217	Carcass Swab	ABF	1185	33	38	44	82	104	35	36	Ery Tet
3630	Carcass Swab	ABF	1469	53	38	30	81	118	35	36	Ery
3635	Carcass Swab	ABF	828	33	39	30	82	104	43	17	Pansusceptible
3637	Carcass Swab	ABF	828	33	39	30	82	104	43	17	Tet
3640	Carcass Swab	ABF	1445	33	39	30	82	104	85	17	Pansusceptible
84	Pig Feces	Conventional	1417	33	39	44	82	104	35	36	Ery
108	Pig Feces	Conventional	1112	33	39	30	82	104	43	68	Tet
461	Pig Feces	Conventional	1440	33	39	46	82	113	35	17	Cip Ery Gen Nal Tet
475	Pig Feces	Conventional	1096	33	38	30	82	104	35	17	Cip Ery Nal Tet
503	Pig Feces	Conventional	1418	33	39	46	174	104	35	17	Ery Tet
524	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Cip Ery Nal Tet
525	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Cip Ery Nal Tet
526	Pig Feces	Conventional	1441	33	38	37	82	104	117	17	Cip Ery Nal Tet
528	Pig Feces	Conventional	1464	33	39	30	82	104	117	17	Cip Ery Nal Tet
533	Pig Feces	Conventional	1465	33	39	30	82	113	117	17	Cip Ery Nal Tet
548	Pig Feces	Conventional	1419	33	38	46	82	104	117	17	Cip Ery Nal Tet
549	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Cip Ery Nal Tet
552	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Cip Ery Nal Tet
553	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Nal Tet
554	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Cip Ery Nal Tet
555	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Cip Ery Nal Tet
556	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Cip Ery Nal Tet
423	Pig Feces	Conventional	1422	33	38	46	82	104	173	17	Ery Tet
429	Pig Feces	Conventional	1423	33	39	46	82	104	47	17	Ery Tet
447	Pig Feces	Conventional	1443	32	38	44	82	113	43	36	Pansusceptible
448	Pig Feces	Conventional	1434	32	38	44	82	104	43	17	Tet

Table 4.1 continued

458	Pig Feces	Conventional	1439	33	38	30	82	104	173	17	Ery Nal Tet
459	Pig Feces	Conventional	1439	33	38	30	82	104	173	17	Ery Nal Tet
623	Pig Feces	Conventional	1442	33	38	132	82	104	85	17	Ery Tet
625	Pig Feces	Conventional	1413	33	38	30	82	104	117	17	Tet
648	Pig Feces	Conventional	1420	32	39	115	115	104	85	17	Tet
653	Pig Feces	Conventional	1466	33	153	30	173	217	44	68	Tet
827	Pig Feces	Conventional	1059	33	153	30	82	104	35	17	Ery Tet
841	Pig Feces	Conventional	1429	33	39	44	82	189	35	36	Ery Tet
856	Pig Feces	Conventional	1445	33	39	30	82	104	85	17	Tet
858	Pig Feces	Conventional	1177	33	38	30	82	104	85	17	Ery
872	Pig Feces	Conventional	1177	33	38	30	82	104	85	17	Ery
1919	Pig Feces	Conventional	1096	33	38	30	82	104	35	17	Ery Tet
3441	Pig Feces	Conventional	854	33	38	30	82	104	43	17	Ery Tet
3451	Pig Feces	Conventional	825	33	39	30	82	113	47	17	Ery
3722	Pig Feces	Conventional	890	33	38	30	82	104	35	36	Nal Tet
3736	Pig Feces	Conventional	854	33	38	30	82	104	43	17	Tet
4246	Pig Feces	Conventional	1056	33	39	30	82	104	43	36	Ery Tet
4396	Pig Feces	Conventional	1430	32	153	30	82	104	44	36	Tet
4489	Pig Feces	Conventional	1417	33	39	44	82	104	35	36	Ery Tet
4490	Pig Feces	Conventional	1438	33	153	30	82	104	44	17	Ery Tet
4507	Pig Feces	Conventional	1096	33	38	30	82	104	35	17	Nal Tet
4511	Pig Feces	Conventional	1096	33	38	30	82	104	35	17	Nal Tet
4516	Pig Feces	Conventional	1425	33	39	134	174	104	43	68	Ch Cip Ery Nal Tet
4517	Pig Feces	Conventional	1425	33	39	134	174	104	43	68	Ch Cip Ery Nal Tet
5409	Pig Feces	Conventional	1134	33	38	30	82	104	173	68	Nal Tet
3480	Carcass Swab	Conventional	890	33	38	30	82	104	35	36	Ery Tet
690	Carcass Swab	Conventional	1421	33	38	44	82	104	117	36	Ery
694	Carcass Swab	Conventional	1435	33	39	134	161	118	43	17	Ery
699	Carcass Swab	Conventional	1443	32	38	44	82	113	43	36	Ery
4324	Carcass Swab	Conventional	1453	32	38	30	167	104	35	17	Ery Tet
4405	Carcass Swab	Conventional	890	33	38	30	82	104	35	36	Nal Tet
4521	Carcass Swab	Conventional	1438	33	153	30	82	104	44	17	Ery Tet
4529	Carcass Swab	Conventional	1096	33	38	30	82	104	35	17	Nal Tet
4540	Carcass Swab	Conventional	1417	33	39	44	82	104	35	36	Ery Tet
892	Carcass Swab	Conventional	1125	33	38	134	161	104	43	17	Ery Tet
1979	Carcass Swab	Conventional	1428	53	38	30	81	118	43	36	Ery

Table 4.1 continued

3490	Carcass Swab	Conventional	1130	33	38	30	82	104	43	68	Tet
3491	Carcass Swab	Conventional	854	33	38	30	82	104	43	17	Tet
3507	Carcass Swab	Conventional	1097	53	38	30	81	104	44	36	Ery Tet
4276	Carcass Swab	Conventional	1123	53	38	44	82	118	35	36	Ery Tet
4338	Carcass Swab	Conventional	1454	58	38	30	167	118	35	17	Ery Tet
4425	Carcass Swab	Conventional	854	33	38	30	82	104	43	17	Ery
4549	Carcass Swab	Conventional	1142	33	153	30	82	104	43	36	Tet
4555	Carcass Swab	Conventional	887	33	38	30	82	104	85	68	Nal Tet
4559	Carcass Swab	Conventional	887	33	38	30	82	104	85	68	Tet
4560	Carcass Swab	Conventional	1455	33	38	37	82	104	85	68	Tet
4562	Carcass Swab	Conventional	887	33	38	30	82	104	85	68	Nal Tet
4563	Carcass Swab	Conventional	887	33	38	30	82	104	85	68	Cip Gen Nal Tet
5472	Carcass Swab	Conventional	1068	33	39	30	78	104	43	17	Ery
4339	Carcass Swab	Conventional	1096	33	38	30	82	104	35	17	Ery Tet
4443	Carcass Swab	Conventional	1428	53	38	30	81	118	43	36	Ery Tet
4444	Carcass Swab	Conventional	1428	53	38	30	81	118	43	36	Ery Tet
4445	Carcass Swab	Conventional	1428	53	38	30	81	118	43	36	Ery Tet

Table 4.2 Sequence Type distribution in the two production systems at farm and slaughter

ST	ABF Farm		ABF Slaughter			Conventional Farm		Conventional Slaughter		
	Nursery	Finishing	Pre-Evisceration	Post-Evisceration	Post-Chill	Nursery	Finishing	Pre-Evisceration	Post-Evisceration	Post-Chill
825		3					1			
828					2					
854	1	2					2		2	
887									4	
890		1		1			1	2		
1055		1								
1056			1				1			
1058				1						
1059							1			
1068	1								1	
1096		2		1		1	3	1		1
1097									1	
1102		1								
1112	3	2		1		1				
1113				1						
1123		2		3					1	
1125									1	
1130	1								1	
1134	1	3				1				
1142									1	
1177		1					2			
1185					1					
1200		1								
1413						8	1			
1414	2									
1415			1							
1416	1			1						
1417						1	1	1		

Table 4.2 continued

ST	ABF Farm		ABF Slaughter			Conventional Farm		Conventional Slaughter		
	Nursery	Finishing	Pre-Evisceration	Post-Evisceration	Post-Chill	Nursery	Finishing	Pre-Evisceration	Post-Evisceration	Post-Chill
1418						1				
1419						1				
1420							1			
1421								1		
1422							1			
1423							1			
1424		1								
1425							2			
1426		1								
1427		1								
1428									1	3
1429							1			
1430							1			
1431	1									
1432		2	1	1						
1433		1								
1434							1			
1435								1		
1436		1								
1437	1									
1438	1						1	1		
1439							2			
1440						1				
1441						1				
1442							1			
1443							1	1		
1444	1									
1445			2		1		1			
1446		2								

Table 4.2 continued

ST	ABF Farm		ABF Slaughter			Conventional Farm		Conventional Slaughter		
	Nursery	Finishing	Pre-Evisceration	Post-Evisceration	Post-Chill	Nursery	Finishing	Pre-Evisceration	Post-Evisceration	Post-Chill
1447		1								
1448		1								
1449				1						
1450	1	2	1							
1451			1							
1452		2								
1453								1		
1454									1	
1455									1	
1463	1									
1464						1				
1465						1				
1466							1			
1467			2							
1468				1						
1469					1					
1470			1							

Table 4.3 Genetic diversity at the MLST loci among 151 *Campylobacter coli* isolates from swine

Locus	Fragment Size (bp)	Number of alleles [a]	Number of variable sites [a]	% Variable sites [a]	d_N /d_S ratio [a]	Pairwise Fst [a]
aspA	477	2 (4)	3 (4)	0.6 (0.8)	0.15(0.08)	0.04
glnA	477	3 (3)	2 (2)	0.4 (0.4)	0.1 (0.66)	0.08
gltA	402	8 (7)	50 (47)	12.4 (11.7)	0.08 (0.07)	0.00
glyA	507	6 (8)	5 (19)	0.9 (3.7)	0.07 (0.05)	0.002
pgm	498	4 (5)	3 (11)	0.6 (2.2)	0 (0.03)	0.03
tkt	459	9 (7)	6 (5)	1.3 (1)	0.05 (0.03)	0.02
uncA	489	3 (3)	2 (2)	0.4 (0.4)	0 (0)	0.00
All Loci	3309	33	69	2.08	0.08	0.02

[a] Comparison of 77 *C. coli* isolates from the antimicrobial free to 74 isolates from the conventional system.

Table 4.4 Unique Sequence Types (STs) that were observed at farm and slaughter of the two production systems

ABF Farm	ABF Slaughter	Conventional Farm	Conventional Slaughter
1102	1113	1413	1097
1112	1185	1418	1125
1200	1449	1419	1142
1414	1451	1420	1421
1415	1467	1422	1428
1424	1468	1423	1435
1426	1469	1425	1453
1427	1470	1429	1454
1431		1430	1455
1433		1434	
1436		1439	
1437		1440	
1444		1441	
1446		1442	
1447		1464	
1448		1465	
1452		1466	
1463			

Table 4.5 Frequency of STs including unique STs and Gene flow analysis between isolates from the ABF and the conventional system

Production Type	Processing Stage	No. of Strains	No. of STs (% Diversity)	No. of Unique STs	Polymorphic sites	Number of Fixed differences	Shared mutations	F_{ST} [b]
ABF [a] (77)	Fecal sample	50	31 (62)	18	71	0	67	0.02
	Carcass swab	27	20 (74)	8				
Conv. (74)	Fecal sample	46	30 (65)	17	90			
	Carcass swab	28	19 (67)	9				

[a] Antimicrobial free production system.

[b] *Campylobacter coli* isolates compared by concatenating seven MLST loci (3309 bp) between the ABF and the conventional system.

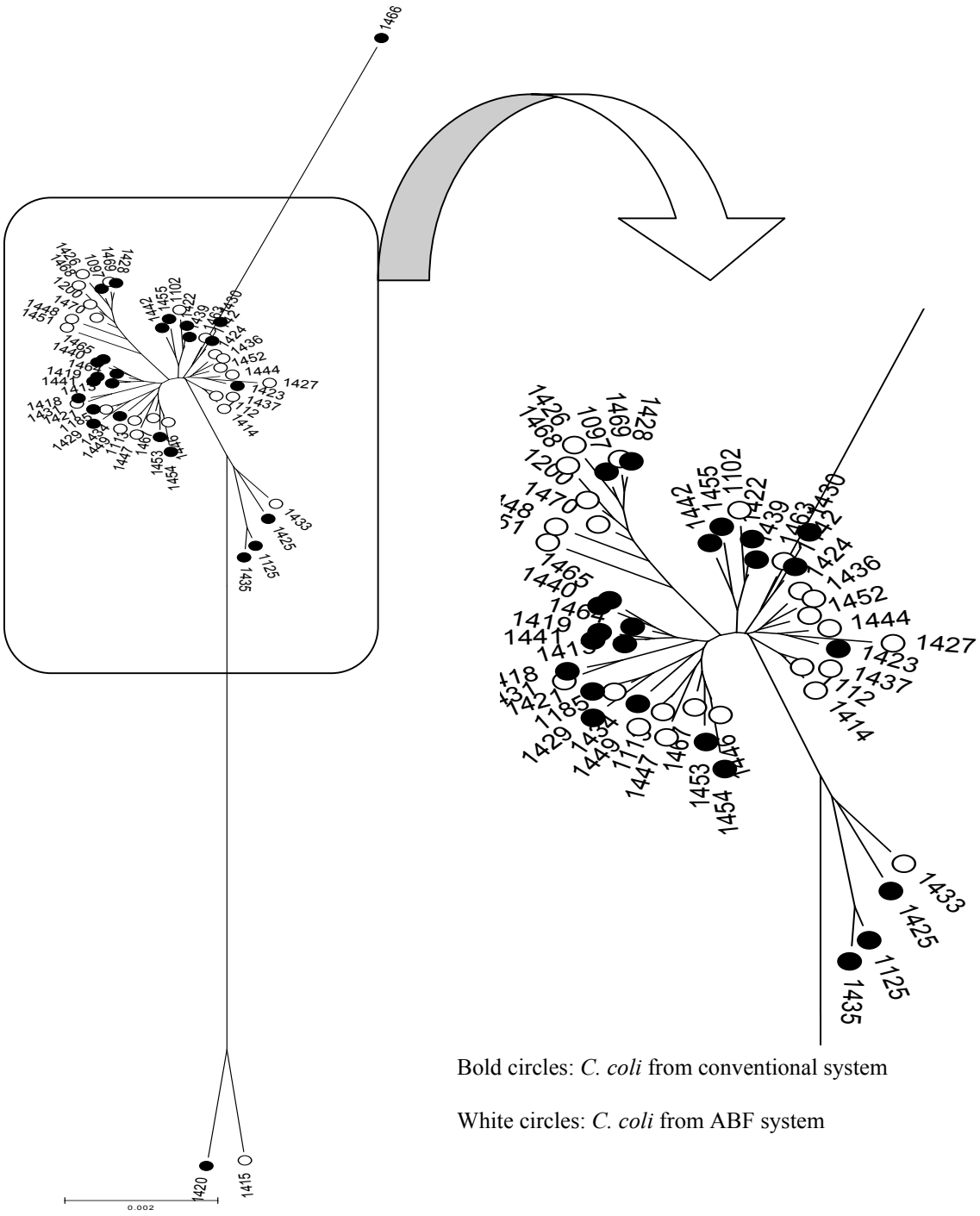

Figure 4.1 Phylogenetic tree constructed using the radial neighbor-joining method using the unique STs from farm and slaughter of the two production systems

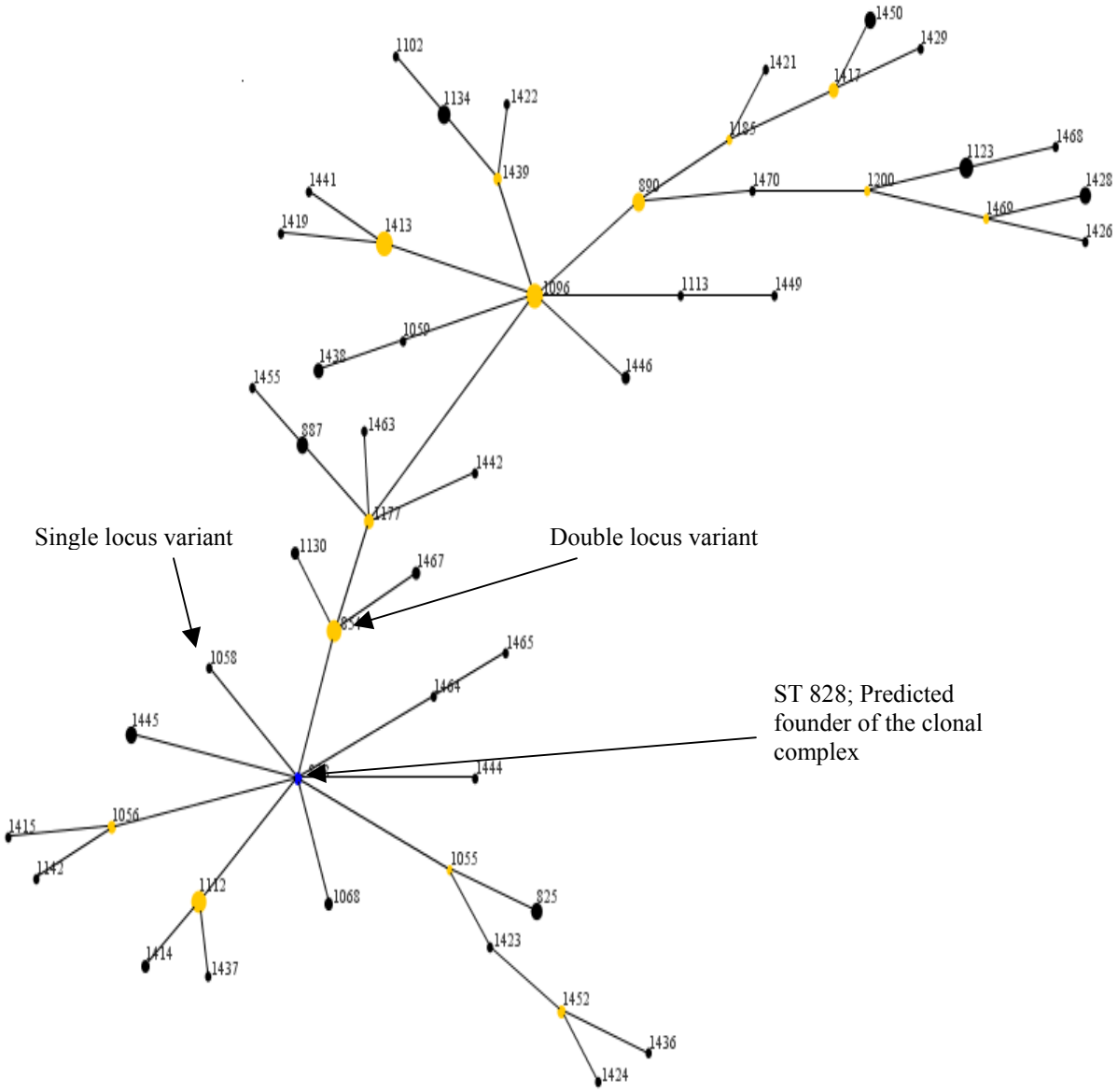

Figure 4.2 *Campylobacter coli* clonal complex generated by eBURST consisting 121 out of the 151 isolates

Table 4.6 eBURST output for 151 Campylobacter coli isolates from the two productions

No. isolates = 151		**No. STs = 74**			**No. re-samplings for bootstrapping = 1000**		
No. loci per isolate = 7		**No. identical loci for group def = 6**					**No. groups = 1**
Group 1: No. Isolates = 124			**No. STs = 53**				**Predicted Founder = 828**
ST	**FREQ**	**SLV**	**DLV**	**TLV**	**SAT**	**Average Distance**	**ST Bootstrap Group**
828	2	9	19	10	14	2.75	54%
1096	9	9	18	15	10	2.55	48%
1177	3	8	17	15	12	2.71	19%
854	7	7	21	15	9	2.61	9%
1058	1	7	19	17	9	2.69	4%
1413	9	7	19	14	12	2.71	8%
1445	4	7	17	15	13	2.84	3%
1464	1	7	17	15	13	2.84	5%
1055	1	7	15	16	14	2.92	3%
1439	2	6	19	15	12	2.76	0%
1444	1	5	17	16	14	2.96	0%
1112	7	5	11	18	18	3.15	0%
1059	1	4	13	21	14	3.01	0%
1130	2	4	13	19	16	3.01	0%
1056	2	4	12	21	15	3	0%
1463	1	4	12	19	17	3.17	0%
887	4	4	10	20	18	3.11	0%
1134	5	4	8	22	18	3.17	0%
890	5	3	16	21	12	2.8	0%
1452	2	3	10	17	22	3.44	0%
1419	1	3	9	22	18	3.25	0%
1417	3	3	6	16	27	3.42	0%
1185	1	3	5	23	21	3.28	0%
1200	1	3	5	3	41	4.19	0%
1469	1	3	2	3	44	5.01	0%
1438	3	2	15	17	18	3.23	0%
1423	1	2	11	17	22	3.46	0%
1441	1	2	10	22	18	3.26	0%
1113	1	2	10	16	24	3.38	0%
1422	1	2	9	23	18	3.3	0%
1436	1	2	9	23	18	3.38	0%
1424	1	2	9	19	22	3.5	0%
1465	1	2	6	17	27	3.61	0%
1437	1	2	6	16	28	3.69	0%
825	4	2	6	15	29	3.69	0%
1470	1	2	5	18	27	3.51	0%
1414	2	2	5	16	29	3.71	0%
1123	6	2	4	7	39	4.67	0%
1428	4	2	1	3	46	5.07	0%
1426	1	2	1	3	46	5.17	0%

Table 4.6 continued

ST	FREQ	SLV	DLV	TLV	SAT	Average Distance	ST Bootstrap Group
1442	1	1	12	19	20	3.3	0%
1068	2	1	10	19	22	3.57	0%
1446	2	1	9	23	19	3.23	0%
1142	1	1	9	19	23	3.32	0%
1467	2	1	8	20	23	3.48	0%
1421	1	1	6	19	26	3.44	0%
1415	1	1	6	17	28	3.61	0%
1455	1	1	6	15	30	3.67	0%
1450	4	1	4	8	39	4.13	0%
1102	1	1	3	9	39	3.96	0%
1449	1	1	2	15	34	3.94	0%
1429	1	1	2	7	42	4.21	0%
1468	1	1	2	6	43	4.82	0%
Singletons: size 21							
1097	1125	1416	1418	1420	1425	1427	1430
1431	1432	1433	1434	1435	1440	1443	1447
1448	1451	1453	1454	1466			

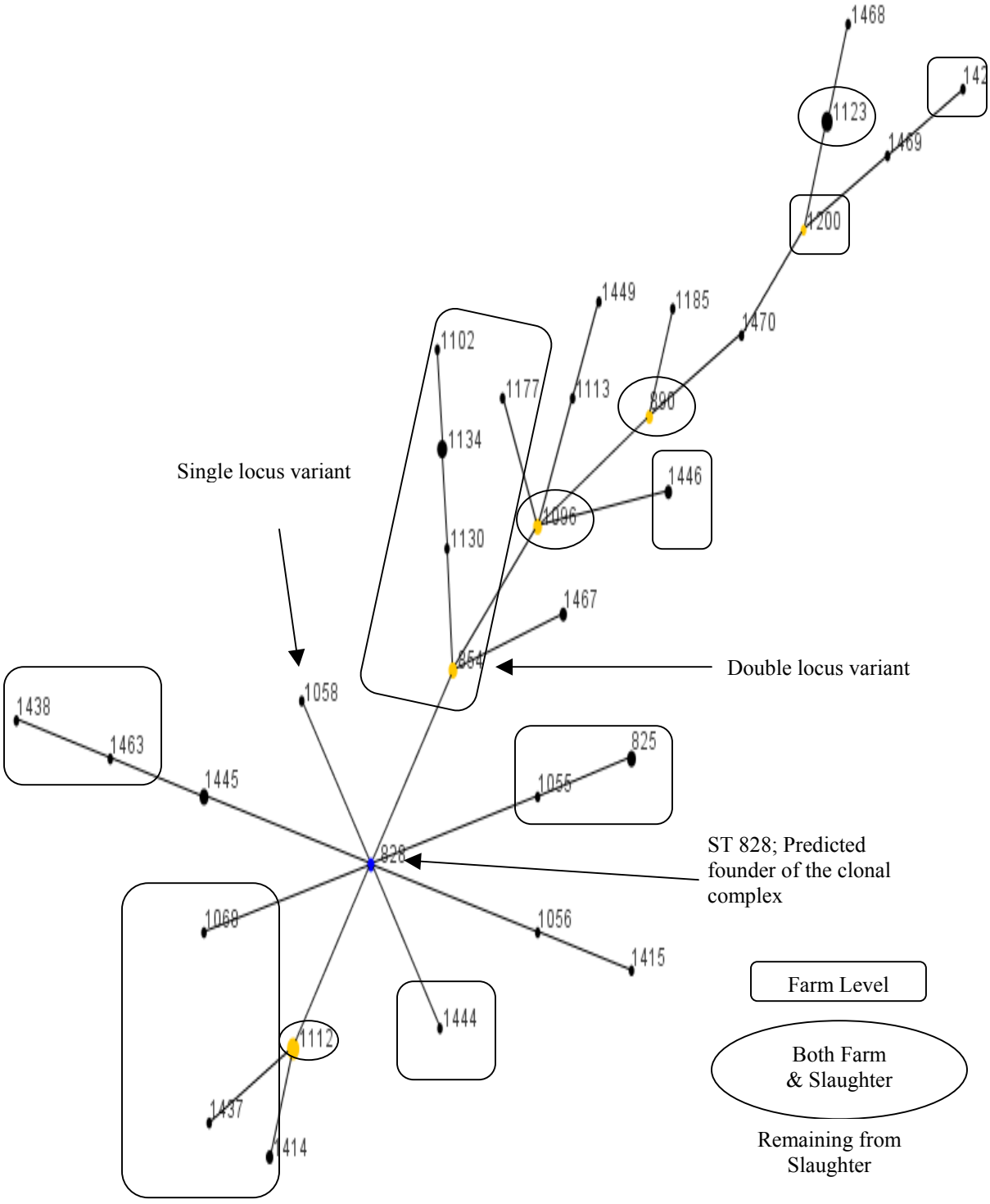

Figure 4.3 *Campylobacter coli* clonal complex for isolates from the ABF system represented by 57 out of the total 77 isolates

Table 4.7 eBURST output for 77 Campylobacter coli isolates from the ABF production

No. isolates = 77		**No. STs = 44**		**No. re-samplings for bootstrapping = 1000**			
No. loci per isolate = 7		**No. identical loci for group def = 6**				**No. groups = 1**	
Group 1: No. Isolates = 57		**No. STs = 32**				**Predicted Founder = 828**	
ST	**FREQ**	**SLV**	**DLV**	**TLV**	**SAT**	**Average Distance**	**ST Bootstrap Group**
828	2	8	10	4	9	2.74	73%
1096	3	6	12	6	7	2.51	41%
1445	3	6	7	9	9	2.93	11%
854	3	5	13	8	5	2.54	9%
1058	1	5	11	9	6	2.7	1%
1055	1	5	8	9	9	3	3%
1112	6	5	8	8	10	3.06	10%
1177	1	4	11	8	8	2.74	1%
1444	1	4	9	9	9	3.03	0%
1056	1	3	10	9	9	2.93	0%
890	2	3	9	13	6	2.7	0%
1130	1	3	9	12	7	2.87	0%
1463	1	3	6	10	12	3.25	0%
1200	1	3	4	2	22	3.93	1%
1113	1	2	7	10	12	3.25	0%
1134	4	2	5	15	9	3.12	0%
1470	1	2	4	10	15	3.35	0%
1414	2	2	4	7	18	3.8	0%
1437	1	2	4	7	18	3.83	0%
1123	5	2	3	3	23	4.64	0%
1469	1	2	2	3	24	4.7	0%
1068	1	1	9	10	11	3.48	0%
1438	1	1	8	10	12	3.35	0%
1446	2	1	6	16	8	3.09	0%
1467	2	1	6	12	12	3.35	0%
825	3	1	4	9	17	3.74	0%
1415	1	1	4	9	17	3.7	0%
1185	1	1	3	12	15	3.41	0%
1449	1	1	1	8	21	4	0%
1102	1	1	1	6	23	3.87	0%
1468	1	1	1	4	25	4.96	0%
1426	1	1	1	3	26	4.93	0%
Singletons: size 9							
1416	1450	1431	1433	1432	1447	1448	1427
1451							

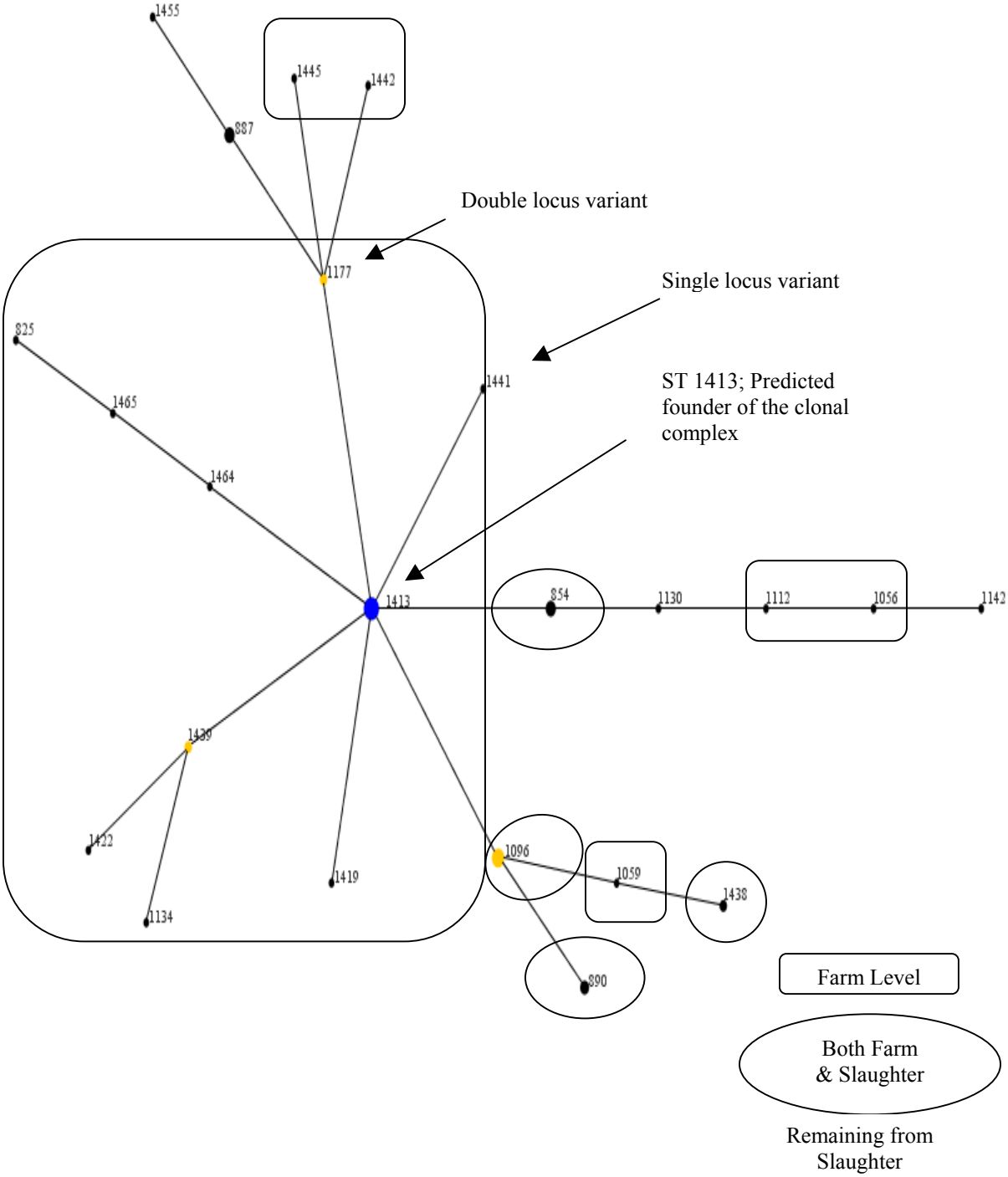

Figure 4.4 *Campylobacter coli* clonal complex generated for isolates from the conventional system represented by 44 out of the 74 isolates

Table 4.8 eBURST output for 74 *Campylobacter coli* isolates from the conventional production system

No. isolates = 74		**No. STs = 43**		**No. re-samplings for bootstrapping = 1000**			
No. loci per isolate = 7		**No. identical loci for group def = 6**					**No. groups = 1**
Group 1: No. Isolates = 47 No. STs = 23							
Predicted Founder = 1413							
ST	FREQ	SLV	DLV	TLV	SAT	Average Distance	ST Bootstrap Group
1413	9	7	10	5	0	1.9	53%
1177	2	7	10	5	0	1.9	55%
1096	6	6	10	6	0	2	13%
1439	2	6	10	6	0	2	16%
854	4	5	14	3	0	1.9	5%
887	4	4	8	8	2	2.36	3%
1130	1	4	8	8	2	2.36	5%
1464	1	3	11	7	1	2.27	2%
1134	1	3	8	9	2	2.45	0%
1419	1	3	6	9	4	2.63	0%
1445	1	2	12	8	0	2.27	0%
1059	1	2	8	11	1	2.5	0%
1441	1	2	8	8	4	2.63	0%
1422	1	2	7	8	5	2.72	0%
1112	1	2	6	10	4	2.72	0%
1056	1	2	5	10	5	2.81	0%
1465	1	2	2	10	8	3.13	0%
890	3	1	10	9	2	2.54	0%
1442	1	1	10	6	5	2.68	0%
1438	2	1	8	12	1	2.59	0%
1142	1	1	6	8	7	2.95	0%
1455	1	1	5	9	7	3.09	0%
825	1	1	2	9	10	3.31	0%
Singletons: size 18							
1440	1418	1423	1443	1434	1420	1466	1430
1425	1421	1435	1453	1125	1428	1097	1123
1454	1068						