ABSTRACT

WRIGHT, SANDRA LASHAWN. Potential Transfer of *Campylobacter* between Turkeys and Swine Produced in Close Proximity, in Eastern North Carolina. (Under the direction of Sophia Kathariou.)

Campylobacter is the leading bacterial cause of human diarrheal cases in the United States and other industrialized nations, with *C. jejuni,* followed by *C. coli,* being the two most frequently implicated species. Contaminated poultry has been identified as a major risk factor for human *Campylobacter* infection. Extensive research on *Campylobacter* has been conducted on broilers; however, limited information exists on pre-harvest colonization of turkeys. The eastern region of North Carolina is a major producer of turkeys, as well as swine. The intertwined turkey and swine operation is a common production system in eastern North Carolina, in which both animals are produced in close proximity, and frequently tended to by the same grower. *Campylobacter* frequently colonizes both turkeys and swine, hence, there is potential for cross-transfer of the organism. Molecular subtyping can be utilized to characterize different strains within a species, and identify strains that are likely shared between different hosts. The sharing of common strains between different animals will likely result in the exchange of exogenous DNA. DNA modification at specific sites (e.g. methylation at GATC sites) is a common feature of bacterial genomes, and frequently associated with restriction-modification systems.

Objectives of the study are (i) to conduct a longitudinal survey on prevalence and antibiotic resistance profiles of *C. jejuni* and *C. coli* from commercial turkey flocks in eastern North Carolina; (ii) to investigate longitudinal trends in fecal prevalence and

antibiotic resistance of *Campylobacter coli* from turkeys and swine, produced in close proximity; (iii) to determine putative genetic relationships between *C. coli* strains from turkeys and swine using molecular subtyping tools; and (iv) to identify possible host-associated attributes (e.g. DNA methylation at GATC sites) that differentiate *C. coli* from turkeys and swine.

Campylobacter was frequently recovered from turkeys, with *C. jejuni* being the predominant species. The prevalence of antibiotic resistance varied between *C. jejuni* and *C. coli*, and in isolates from birds of different ages, with marked differences in resistance patterns being observed. *C. coli* was highly prevalent in all swine herds involved in the study; however, high prevalence in swine was not always accompanied by high prevalence in the corresponding paired turkey farms. Host-associated trends in prevalence of antibiotic resistance were detected, and varied with flock age (a temporal trend was not seen in swine). Strain subtyping of turkey- and swine-derived isolates was performed using multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) with two enzymes, and *fla*-typing. Host-associated subtypes were detected with all three subtyping tools. Importantly, common strain types from turkeys and swine were not observed with MLST and/or PFGE. DNA modification systems specific for adenines (resistance to *Mbo*I digestion) at GATC sites were detected in *C. coli* from swine but not in turkeys. Swine-associated STs were observed in several of the strains with *Mbo*I-resistant DNA.

Based upon all findings, factors such as bacterial prevalence, antibiotic resistance, genetic relationships, and distinct genetic attributes appear to be host-specific. The lack of commonality, phenotypically or genotypically, between turkeys and swine suggests

that transfer of *Campylobacter* between hosts is limited, and therefore was below the detection limit of this study, or operates under special circumstances.

POTENTIAL TRANSFER OF *CAMPYLOBACTER* BETWEEN TURKEYS AND SWINE PRODUCED IN CLOSE PROXIMITY, IN EASTERN NORTH CAROLINA

by
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DEDICATION

All of the hard work put into my research is dedicated to the one person who makes my life worth living and makes all things believable, Joaquin Shamese Leak. You have stood beside me through this two and a half year journey in my life. You have been my strength on those tired days, my motivation on those days of despair, my light on those days when I could not find my way and felt lost, my joy on those days when I could not find an ounce of happiness to produce a smile. You are my answer for everything because on those days stricken with grief and frustration I would turn to you and things would make sense and life would have so much more meaning. I know that all I do I do for you. You are someone so special and have brought more good to my life than you will ever know. A gift specially wrapped for me from God. To this day I believe God sent you to me, not as a hindrance but as a means to succeed. He bestowed upon me a love that is indescribable, the reason why I strive is because you deserve the best. You are my greatest accomplishment, an achievement that can never be surpassed. I love you baby, you are the beat to my heart. And remember without a heartbeat, there is no life.

BIOGRAPHY

Sandra LaShawn Wright, daughter of Cassandra Ann Wright and Raymond Cornelius Easterling, was born in Laurinburg, North Carolina (Scotland County) on March 21, 1980 in Scotland Memorial Hospital. She is the eldest of her mother's three children, Ricardo Wright (24) and Jamecia Wright (21). She was raised, loved, and nurtured by her loving grandmother, Annie Wright. She maintained exceptional scores throughout her primary and secondary school years, and received numerous awards for academic achievement. At the start of her secondary school years at I. Ellis Johnson Middle School, she was enrolled in the AG (Academically Gifted) program. During high school she was a member of the Beta Club and National Honors Society. Throughout high school she participated in extracurricular activities such as volleyball, softball, and track and field. She graduated from Scotland High School in May 1998, with academic honors. Because of her aspirations to be a veterinarian, she applied to North Carolina State University (providing the only veterinarian program in the state). Upon acceptance into the university she focused her interest on the Animal Science program; however, pursued a dual major in Animal Science and Food Science in 2000. She was an honoree of the Dean's List at the completion of her first semester, and was involved in various recreational activities, club organizations, and volunteer-based programs. She received a B.S. degree in both Animal Science and Food Science in May 2003. On September 18th of the same year, she gave birth to a beautiful baby boy, Joaquin Shamese Leak (the love of her life). She worked as a full-time research techinician in Dr. Sophia Kathariou's food microbiology laboratory for one year after graduation. Dr. Kathariou blessed her with the opportunity to further her education and pursue a M.S. degree in Food Science.

Not really knowing how far this opportunity would take her, she accepted the offer and conducted her own research project on the potential transfer of *Campylobacter* between turkeys and swine produced in close proximity. In December 2006 she completed all requirements for a M.S. degree, and now knows that if you believe you can achieve.

"Life was never promised to be easy, but it is "do"-able." -Sandra Wright

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Mommy. Mommy you helped me to stay grounded and humble throughout my life with your prayers and perpetual love. And last but not least, my darling child Joaquin. You may never understand what having you in my life at this time in my life really meant to me but just know that you were a blessing in disguise. Finishing school and raising you are two achievements in one. The ability to do so was truly a blessing. God has looked over me and guided me along the way. To all that made a difference in my life, I thank you and know that words can never express my gratitude.

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1. Review of literature on *Campylobacter*

1.1 HISTORICAL EMERGENCE

It has only been in the last 30 years that *Campylobacter* spp. have been recognized as a major cause of human illness. The organisms were considered to be an opportunistic human pathogen due to their rare association with human blood cultures in the late 1950's (Moore et al, 2005). In the 1880's, Theodore Escherich made a possible discovery of *Campylobacte*r in the stools of German infants with diarrheal illness (Moore et al, 2005). Microbiologists McFadyean and Stockman (1913) made a putative identification of *Campylobacter* in association with sheep abortions. Smith (1918) confirmed their results upon isolation of similar organisms from aborted fetuses of cattle. Due to the spiral appearance of the organism, Smith assigned it to the genus *Vibrio*, hence naming it *Vibrio fetus*. Jones et al (1931) determined that the jejunum of diseased cattle was the first site to be infected by the "vibrios", hence, they were termed *Vibrio jejuni*. Vibrios isolated from the colon of swine with dysentery were given the name *Vibrio coli* (Doyle, 1944; Doyle, 1948).

Vinzent et al (1947) reported the first human case in association with microaerophilic vibrios involving a pregnant woman, whose unborn child died and these organisms were isolated from the fetus. Elizabeth King (1957, 1962) proposed two different types of enteritis-associated vibrios, one being *V. fetus* (optimal growth at 37°C) and the other being a thermophilic species that could grow at an optimal temperature of 42°C. Dekeyser et al (1972) devised an isolation procedure in which stool specimens were filtered through 0.64-0.65 μm membranes onto agar medium. The isolation technique was a step toward improvement, but it was too cumbersome. Skirrow (1977)

described a more direct technique for isolating campylobacters from stool specimens, which involved directly plating fecal matter onto blood agar medium containing selective antibiotics that prohibited the growth of competing microflora but permitted *Campylobacter* growth. Improvements in isolation methods have led to the recognition of *Campylobacter* as the most frequent bacterial cause of human gastroenteritis and have confirmed its significance in regards to food safety and public health.

1.2 TAXONOMY

In the early 1900's, unbeknownst as *Campylobacter* the organism was given the name *Vibrio fetus*, due to its spiral shape. Over time it was recognized that the microaerophilic vibrios were not as closely related to other species within the genus *Vibrio* as once believed, because the organisms were not able to oxidize or ferment sugars and had a different G+C content (~34%) than *Vibrio cholerae* (~47%) (Moore and Matsuda, 2002). Growth temperature requirements were also dissimilar between the two: *V. fetus* required microaerophilic conditions, whereas *V. cholerae* was a facultative anaerobe (Moore and Matsuda, 2002).

Sebald and Veron (1963) proposed the genus *Campylobacter* with *C. fetus* (Smith and Taylor, 1919) as the type species. The genus *Campylobacter* was grouped together with genera such as *Spirillium* and *Aquaspirillium*. However, Krieg and Hyelemon (1976) determined that the genera *Campylobacter* and *Spirillium* were not closely genetically related. Veron and Chatelain (1973) re-named the "related vibrios", originally *V. jejuni* (Jones et al, 1931) and *V. coli* (Doyle, 1948) as *C. jejuni* and *C. coli*, respectively. Both organisms were grouped under *Campylobacter fetus* subsp. *jejuni* by

Smibert (1978). The family *Campylobacteraceae* was proposed by Vandamme et al (1991), and included the closely related genera *Campylobacter* and *Arcobacter*.

1.3 BACTERIOLOGY AND PHYSIOLOGY

Campylobacter are slender, spiral curved rods, 0.2-0.9 μm in width and 0.5-5 μm in length. The organisms are gram-negative, non-sporeformers that become coccoid (or spherical) when the cultures have aged or have been exposed to air for a period longer than they can withstand. In *C. jejuni* cultures this stationary-phase morphological conversion from spiral-bacillary to coccoid forms is characterized by the loss of culturability (Kelly, 2001). Harvey and Leach (1998) described the coccoid appearance as a form of degeneration due to oxidation, and not as an active process. *Campylobacter* cells are highly motile with a characteristic corkscrew-like movement due to the presence of a single polar unsheathed flagellum located at one or both ends. There are some nonmotile species such as *C. gracilis*, and others that have multiple flagella (*C. showae*).

Campylobacter are microaerophilic, meaning the organisms require an environment with a low level of oxygen (3 to 5%) for optimal growth; however, they prefer a relatively high level of carbon dioxide (2 to 10%). *Campylobacter* have a respiratory type of metabolism. There are several species that require an anaerobic environment for optimal growth, or require fumarate with formate or hydrogen in order to grow in the presence of low oxygen concentrations. *In vivo*, anaerobic growth could be a significant factor in the intestinal colonization of *Campylobacter* and its ability to induce illness (Kelly, 2001). Organisms cannot hydrolyze gelatin, casein, starch, or tyrosine. Except for *C. gracilis*, oxidase activity is present in all other species; however, there is no lipase or lecithinase activity.

In regard to humans and animals, there are pathogenic *Campylobacter* spp., which can be found in the gastrointestinal tract, reproductive organs, and oral cavity. The low G+C content of the *Campylobacter* genome ranges from 32-35% (Nuijten et al, 1990), with *C. jejuni* and *C. coli* having an average G+C content of 31.8% and 32.6%, respectively (Owen and Leaper, 1981). Currently, there are 14 validated *Campylobacter* species, with *C. fetus* (Smith and Taylor, 1919) being the type species.

Campylobacter jejuni and *C. coli* are the most significant campylobacters from the perspective of food safety and public health, due to their association with human enteric disease. *C. jejuni* has two subspecies, *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. The difference between the two subspecies is that the latter does not possess nitrate reduction and/or catalase activity. Differentiation between *C. jejuni* and *C. coli* has been problematic from a taxonomic standpoint because the two species are highly similar, phenotypically and genotypically. The hippurate hydrolysis test has been the most reliable tool in differentiation of the two species, due to the absence of hippuricase activity in *C. coli*. The genomes of *C. jejuni* and *C. coli* are approximately 1.7 Mb in size (Chang and Taylor, 1990), which is about one-third of the *E. coli* genome.

1.4 ENVIRONMENTAL STRESS RESPONSE OF *CAMPYLOBACTER*

1.4.1 Introduction

There are a multitude of environmental stresses, which make it highly unlikely for *C. jejuni* and *C. coli* to survive outside of the host for prolonged periods of time. Due to poor survivability in the environment, *Campylobacter* spp. must have defense mechanisms to respond to varying external stimuli. Park (2002) compared *Campylobacter* spp. to other foodborne pathogens, and noted the fragility of the

organisms when exposed to air, inability to propagate outside of a host, and extreme sensitivity to many different environmental conditions. *Campylobacter* spp. have developed response mechanisms to overcome environmental stresses and cause illness in humans. However, little is known about the survival mechanisms of *Campylobacter* that allow the organisms to withstand certain stresses and thrive in an environment (e.g. food, animals).

Park (2002) described the limited ability of *C. jejuni* NCTC 11168 to regulate gene expression in response to environmental stresses. *C. jejuni* lacks important regulators to defend against stresses such as oxidative stress, cold shock and heat shock. However, *C. jejuni* contains several heat shock proteins, two negative regulators, and a two-component regulatory system as stress defenses (Murphy et al, 2006).

1.4.2 Viable but nonculturable state of *Campylobacter*

Limited nutrients and entry into a stationary phase are causative factors of the viable but nonculturable (VBNC) state of *Campylobacter* (Murphy et al, 2006). In the VBNC state, *Campylobacter* cells transition from their spiral form to a coccoid form. This does not appear to be an active process, because there is no change in the profiles or synthesis of proteins during the transition (Hazeleger et al, 1995). *Campylobacter* cells could revert from coccoid to their spiral forms following transit through animals (Rollins and Colwell, 1986). Pearson et al (1993) described the ability of nonculturable forms of *Campylobacter* spp. from water sources to colonize chickens after consumption of the water. Such a study suggests the potential resuscitation of the VBNC forms of *Campylobacter* spp.

Studies have investigated the resuscitative VBNC forms of *Campylobacter* in animals (Saha et al, 1991; Stern et al, 1994) and aquatic environments (Thomas et al, 2002), and when under stress-related conditions (Chaveerach et al, 2003). The ability of VBNC cells to colonize animals may vary from animal to animal and between *Campylobacter* strains. An environment with unfavorable conditions is the probable cause of the VBNC state of *Campylobacter*. *Campylobacter* may have the potential to survive in cold environments for longer periods of time when in a VBNC state than detected by culturability (Murphy et al, 2006). Lazaro et al (1999) described the use of indicators to detect viability of *Campylobacter* cells during long-term storage at temperatures of 4˚C and 20˚C, and detected viability for several months at 4˚C.

1.4.3 Two-Component Regulatory Systems

Regulatory mechanisms assist in controlling gene expression when there is a change in the environment. In bacteria, two-component regulatory systems are involved in responses to environmental changes via regulating sets of specific genes. Two proteins, a sensor protein and a response regulator protein, are involved in these systems. The sensor proteins provide external sensing, while response regulators function as effectors to respond to changes in environmental stimuli (Murphy et al, 2006). Stock et al (2000) described a two-component signal transduction system (TCS), which consist of a sensory histidine kinase (HK) located in the cytoplasm and a response regulator (RR) in the cytoplasmic membrane.

The genome of *C. jejuni* 11168 has nine putative RRs, six HKs, and five TCSs accompanied with a HK and RR (Parkhill et al, 2000). *Campylobacter* colonization is mediated by three of its five two-component regulatory systems (Murphy et al, 2006).

Bras et al (1999) identified the RacR-RacS system as an important system involved in temperature-dependent growth and colonization of *Campylobacter* in the intestinal tract of chickens. A mutation in the *racR* gene (RR) reduced the ability of *Campylobacter* to colonize the chicken intestinal tract, resulting in temperature-dependent changes in its protein profile and growth characteristics (Bras et al, 1999).

1.4.4 Other Survival Mechanisms

There are other survival mechanisms that may assist in the response and adaptation of *C. jejuni* and *C. coli* to external stimuli. Several studies have investigated biofilm formation as a potential stress defense mechanism (Somers et al, 1994; Buswell et al, 1998; Trachoo et al, 2002), in which *C. jejuni* could tolerate certain stresses (e.g. presence of oxygen, cold temperatures) when a biofilm was formed. Trachoo et al (2002) indicated the increased viability of *C. jejuni* in biofilms, whereas Dykes et al (2003) described a reduction in the survival rate of a pathogenic *C. jejuni* strain in a biofilm.

C. jejuni lacks the *rpoS* gene that mediates the stress response mechanism for the transition of *Campylobacter* cells into stationary phase (Parkhill et al, 2000). Due to the absence of the RpoS stationary phase response mechanism, *Campylobacter* cells in stationary phase were more susceptible to different environmental stresses than mid-exponential phase cells (Kelly et al, 2001). In stationary phase, alterations in protein synthesis will affect the expression of genes involved in bacterial protection against different stresses (Hengge-Aronis, 1996).

1.5 EPIDEMIOLOGY

According to the Centers for Disease Control and Prevention, *Campylobacter* is the leading bacterial cause of acute human gastroenteritis in the United States. The

incidence of disease is about 15 cases per 100,000 people diagnosed in the US, resulting in nearly 2.4 million cases annually. The thermophilic species, *Campylobacter jejuni* and *coli*, are the primary etiologic agents of human enteric cases and are clinically indistinguishable of one another. Human illness is more frequently caused by *C. jejuni* (85% or more), whereas *C. coli* (5-10%) has also been implicated, but less frequently (Friedman et al, 2000).

Most illnesses caused by *Campylobacter* occur sporadically, and not as part of outbreaks. The occurrence of *Campylobacter* infections is more frequent during the summer than in the winter, possibly due to the greater consumption of poultry products that have not been handled or cooked properly (Friedman et al, 2000). The impact of seasonality on the prevalence of *Campylobacter* in market weight poultry flocks has been investigated (Willis and Murray, 1997; Patrick et al, 2004). As the weather became warmer, the incidence of human *Campylobacter* infections increased, as well as flock positivity (Patrick et al, 2004).

The clinical symptoms of campylobacteriosis include fever, abdominal cramps and/or pain, and diarrhea (often bloody). Bloody diarrhea can be accompanied by nausea and vomiting. The onset of symptoms occurs within two to five days after exposure. A typical case of illness generally lasts about a week (Moore et al, 2005). Campylobacteriosis is a self-limiting disease, therefore the ill frequently do not seek further medical attention. However, relapses may occur in 20% of patients who do not receive and/or seek treatment (Blaser, 1997). Death is rare; however, it has been estimated that 100 people will die from the disease each year in the United States (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/campylobacter_g.htm).

All age groups are at risk of infection; however, the most susceptible are infants, the elderly, and immunocompromised. *Campylobacter* infection occurs more frequently in developing than in developed countries, however, clinical features of infection differ (Blaser, 1997). Young adults are the most affected age group in developed countries, whereas young children (<2 years old) are predominantly affected in developing countries (Blaser, 1997). Only a small number of *Campylobacter* organisms are necessary to cause an infection. Black et al (1988) described an infectious dose of <1000 organisms to cause illness. In another study, Robinson (1981) determined via ingestion that 500 cfu/ml in milk could result in illness.

CDC began a national surveillance program in 1982 to gain more insight on the cause and dissemination of disease due to *Campylobacter* infection (http://www.cdc.gov/ncidod /dbmd/diseaseinfo/campylobacter_g.htm). In 1996 a more detailed active surveillance system was implemented as an informative means of determining how the disease occurred and what factors contributed to the risk of becoming ill. Continuing efforts are made by the CDC to inform the public about *Campylobacter* infections and ways to prevent becoming infected.

1.6 CLINICAL ASPECTS OF *CAMPYLOBACTER*-RELATED ENTERITIS

1.6.1 Gastroenteritis

Globally, thermophilic *Campylobacter* spp. (*C. jejuni* and *C. coli*) are the primary etiologic agents of human gastroenteritis. *C. jejuni* is responsible for 80-85% of all enteric *Campylobacter* infections, whereas, *C. coli* accounts for 10-15% (Moore et al, 2005). The occurrence of *Campylobacter* infection has been reported from a multitude of countries, including the United States (Koehler et al, 2006), Denmark (Olesen et al,

2005), Czech Republic (Prikazska et al, 2004), Sicily (Scarlata et al, 2004), Finland (Schildt et al, 2006), Australia (Sinclair et al, 2005), and Barbados (Workman et al, 2006).

Diarrhea is the most closely associated symptom of campylobacteriosis, being either watery or bloody. Reports have identified diarrhea as the predominant symptom in clinical patients (Sojo et al, 1982; Otasevic et al, 2004). Clinical studies have indicated the presence of blood in the stools of 78-90% of the patients involved (Anders et al, 1981; Sojo et al, 1982; Ali et al, 2003). A study conducted by Tracz et al (2005) suggested the clinical relevance of the plasmid pVir in *C. jejuni* infections due to its association with bloody diarrhea. There was no correlation between the plasmid and a higher incidence of clinical symptoms; however, the plasmid marked an invasive infection. Other symptoms of *Campylobacter*-related enteritis are abdominal pain, fever, and vomiting (Aristegui et al, 1987; Drioueche et al, 1989; Ali et al, 2003; Otasevic et al, 2004; Scarlata et al, 2004).

Generally, *Campylobacter* infections can affect all age groups, however, clinical manifestations are age-dependent. Based on report findings, *Campylobacter* is frequently implicated as a causative agent of diarrheal illness among children (Anders et al, 1981; Aristegui et al, 1987; Ali et al, 2003; Koehler et al, 2006; Workman et al, 2006). Thermophilic campylobacters accounted for ~95% of the cases in the study conducted by Workman et al (2006).

It is likely for *Campylobacter* infection to occur during the early stages of infancy. Anders et al (1981) reported the onset of symptoms in neonates between two to eleven days of age; however, the level of illness severity was low. Findings suggested

transmission from mother to child during the time of birth or shortly thereafter. Factors that may influence the duration of diarrheal illness in infants were discussed by Househam et al (1990). Nourishment may play a role in the prolonged status of disease associated with diarrhea. An under-nourished infant (<3 months of age) is less likely to have a self-limiting disease, as would a well-nourished infant over six months of age (Househam et al, 1990).

1.6.2 Systemic Infection

Due to the invasiveness of *Campylobacter*, infection may spread to the bloodstream and lead to a more systemic infection. The actual occurrence of bacteremia resulting from acute gastroenteritis is unknown. Although reports describe bacteremia in patients infected by *C. jejuni*, bacteremia is not generally reported (Peterson, 1994). Bacteremia is detected in less than <1% of patients with campylobacteriosis, occurring more frequently among individuals in a severely immunocompromised state (Butzler, 2004; Crushell et al, 2004). The immunity of this particular at-risk population is extremely weakened, therefore, the body's defenses are not strong enough to protect against invasive pathogens.

A study conducted by Sorvillo et al (1991) investigated the incidence and characteristics of *Campylobacter* infection among AIDS patients in Los Angeles County. AIDS patients without *Campylobacter* had a higher survival rate (21 months) than patients with *Campylobacter* infection (14 months), with a higher rate of bacteremia in AIDS patients with *Campylobacter* infection being noted. Pigrau et al (1997) indicated that the occurrence of bacteremia due to *Campylobacter* infection in AIDS patients from

Spain was 0.8%. In patients with advanced HIV or AIDS, bacteremia was found to be persistent or recurrent, despite appropriate antimicrobial treatment (Crushell et al, 2004).

Bacteremia was diagnosed in 15 Danish patients, with *C. jejuni* and *C. coli* accounting for a majority of the cases (Schonheyder et al, 1995). Most of the patients (11/15) had underlying illnesses, indicating the significance of immunity to fight off infections. Ladron de Guevara et al (1994) described several cases of bacteremia caused by *Campylobacter* infection, ranging in age, gender, and state of illness. The findings suggest that any individual who experiences diarrheal illness from *Campylobacter* infection are at risk of bacteremia.

1.6.3 Guillain-Barre Syndrome (GBS)

Although *Campylobacter* infection is a self-limiting disease, there are instances where serious complications may arise. In most cases, Guillain-Barre Syndrome (GBS) is frequently preceded by a number of infectious bacterial and viral processes (Nachamkin, 2002). GBS is the common post-infectious manifestation of *Campylobacter*-related enteritis. GBS is an acute demyelinating disease affecting the peripheral neurons and is characterized by an ascending paralysis (Moore et al, 2005). The presence of activated T lymphocytes and antibodies against peripheral nerve myelin suggests an autoimmune pathogenesis (Hadden and Gregson, 2001). Typically, the onset of GBS symptoms is one to three weeks after the onset of *C. jejuni* infection (Butzler, 2004). Limb weakness is the most pronounced clinical feature of GBS. Weakness progresses over a period of days and up to four weeks, more frequently in an ascending pattern from the lower to upper limbs (Cosi and Versino, 2006). Other symptoms may include sensory disturbance, numbness, pain, bladder problems, and difficulty

swallowing (Hadden and Gregson, 2001). Typically, the infective agent has been eradicated before the onset of neurological symptoms (Hadden and Gregson, 2001).

Hadden and Gregson (2001) summarized the studies that have reported an association between preceding *Campylobacter* infection and GBS in Australia (Kaldor and Speed, 1984), Belgium (Boucquey et al, 1991), China (Ho et al, 1995; Yuki et al, 1999), Germany (Enders et al, 1993; Von Wulffen et al, 1994), Italy (Guarino et al, 1998), Japan (Kuroki et al, 1993; Hao et al, 1998, Koga et al, 1999; Kuwabara et al, 2004), the Netherlands (Jacobs et al, 1996), the United Kingdom (Gregson et al, 1993; Rees et al, 1995), and the United States (Ropper, 1988; Gruenewald et al, 1991; Mishu et al, 1993).

Although the antecedent to GBS, *C. jejuni* infection occurs more commonly than does GBS, thus the risk of post-infectious GBS is relatively low (Nachamkin et al, 1998). It is estimated that one out of every 1000 cases of *C. jejuni* infection will be followed by GBS (Nachamkin et al, 1998). Studies have identified certain serotypes in association with *C. jejuni* infection followed by GBS (Enders et al, 1993; Kuroki et al, 1993; Hao et al, 1998), however, no specific serotypes were associated with GBS in clinical isolates from the United Kingdom (Rees et al, 1995). *C. jejuni* belonging to the Penner serotype 19 was isolated from 83% and 77% of patients, respectively (Kuroki et al, 1993; Hao et al, 1998). The Lior serotype 11 was predominant in German patients with GBS, albeit Lior 4 was the most common serotype associated with enteritis in Germany (Enders et al, 1993).

Gangliosides are constituents of the myelin sheath, which encloses axons and nerve fibers. Ganglioside GM1 has a wide distribution in the nervous system and other

tissues (Hadden and Gregson, 2001). They have multiple effects on signal transduction, and interact with ion channels, growth factors receptors, and adhesion molecules (Hadden and Gregson, 2001). GM1 is the ganglioside that is most frequently implicated in association with GBS (Hadden and Gregson, 2001). The antibodies (IgA, IgG, and IgM) to ganglioside GM1, as well as other minor gangliosides, have been associated with GBS. Gregson et al (1993) and Jacobs et al (1996) detected anti-GM1 antibodies in 58% and 32% of *Campylobacter*-associated GBS patients, respectively.

Reports have identified the involvement of all three antibodies in GBS cases preceded by *Campylobacter* infection (Kaldor and Speed, 1984; Enders et al, 1993; von Wulffen et al, 1994). Japanese studies have reported two different anti-ganglioside antibodies in the sera of GBS patients. Koga et al (1999) indicated that serological evidence confirmed a preceding history of diarrheal illness in 85% of patients with IgA anti-ganglioside antibodies. Ho et al (1995) and Yuki et al (1999) found that IgG anti-ganglioside antibodies were most frequent in clinically defined GBS cases involved in their studies, 66% and 47% of the patients were seropositive for *C. jejuni* infection, respectively.

1.7 RESERVOIRS

Campylobacter are widely distributed in nature, and a plethora of animals have been recognized as reservoirs, ranging from domesticated animals to wild birds and rodents. *C. jejuni* is a zoonotic pathogen, making it easier for the organism to gain access to the human food supply. *Campylobacter* can form both commensal and pathogenic associations with animals. Commensal poultry and meat animals are typically asymptomatic carriers of *Campylobacter* infections, frequently colonizing the cecum of

poultry (Newell and Fearnley, 2003) and the digestive tract of swine (Harvey et al, 1999). A high prevalence of thermophilic *Campylobacter* spp. in poultry and swine has been reported (Rosef et al, 1983; Harvey et al, 1999; Cox et al, 2000; Wesley et al, 2005).

Many animals pose as reservoirs for infection, including wild and domestic birds (Aydin et al, 2001; Waldenstrom et al, 2002; Lillehaug et al, 2005; Tsai and Hsiang, 2005), cattle (Stanley and Jones, 2003; Halbert et al, 2006; Minihan et al, 2006), sheep (Stanley et al, 1998; Zweifel et al, 2004), and domestic pets such as dogs, cats, and hamsters (Gebhart et al, 1989; Hald et al, 2004a; Bender et al, 2005; Workman et al, 2005; Acke et al, 2006).

Other potential reservoirs of *Campylobacter* infection are shellfish (Wilson and Moore, 1996; Endtz et al, 1997) and produce (Brandl et al, 2004; Karenlampi and Hanninen, 2004), which could also be contaminated by animal feces or contaminated water. Water sources have also been implicated (Hanninen et al, 1998; Daczkowska-Kozon and Brzostek-Nowakowska, 2001; Zimmer et al, 2003) as well as milk (Warner et al, 1986; Harris et al, 1987; Schildt et al, 2006).

C. jejuni can maintain a viable but non-culturable (VBNC) state in water (Rollins and Colwell, 1986), in which microbial recovery in the laboratory is unlikely. Results regarding VBNC infectivity have been obtained in different studies (Saha et al, 1991; Stern et al, 1994; Ziprin and Harvey, 2004). Saha et al (1991) reported the resuscitation of injured, nonculturable *C. jejuni* cells via passage through a susceptible host. Stern et al (1994) indicated that nonculturable *Campylobacter* were able to colonize chicks; however, Medema et al (1992) noted the inability of VBNC *C. jejuni* to colonize one-day

old chicks. Likewise, Ziprin and Harvey (2004) reported the inability of VBNC *C. jejuni* cells to revert to a culturable form to colonize chicks.

The vehicles of *Campylobacter* infection have changed substantially over the years. Spanning almost a decade (1978-1987), water and unpasteurized milk were the primary vehicles of infection; however, foods were the implicated source of over 80% of outbreaks from 1988-1996 (Friedman et al, 2000).

1.8 *CAMPYLOBACTER* COLONIZATION IN TURKEYS

Extensive research has investigated the occurrence of *Campylobacter* in poultry; however, more frequently in broiler flocks. Likewise, the association between *Campylobacter* infection and turkeys is being studied more readily. As a member of the avian species, turkeys have been found to be frequently colonized with *Campylobacter*. *Campylobacter* resides as a commensal organism in the animal host (Newell and Fearnley, 2003). Turkey production is an intensively reared large-scale system, which could potentially facilitate the dissemination of *Campylobacter* infection in a closed environment. Luangtongkum et al (2006) described the high prevalence of thermophilic *Campylobacter* in organic and conventional turkey production systems. Commercial poultry are the major natural reservoirs of *C. jejuni* (Sahin et al, 2002), thus, perpetuating the incidence of human infections. Most cases of infection are due to the handling of raw poultry or the consumption of raw or undercooked poultry.

Carriage rate is a pertinent factor in establishing a relationship between flock colonization and the occurrence of *Campylobacter* infection in humans. High turkey flock positivity has been reported, with the prevalence of *Campylobacter* ranging from 65-95% (Cox et al, 2000; Wesley et al, 2005; Luangtongkum et al, 2006). Turkeys are

asymptomatic carriers, albeit high carriage rates are frequent in poultry. Exclusively, thermophilic campylobacters have been identified as the commensal organisms of poultry, with *C. jejuni* being the predominant species (Wallace et al, 1998; van Looveren et al, 2001; Borck et al, 2002; Luber et al, 2003). However, other studies have isolated *C. coli* more frequently from turkey flocks (Smith et al, 2004; Lee et al, 2005).

Wallace et al (1998) studied the colonization rate of thermophilic *Campylobacter* species in turkey poults. All birds were negative upon arrival; however, by day 21 after placement, the carriage rate was 100%, with the number of organisms increasing with distance from the beak (the highest being found in the ceca) (Wallace et al, 1998). Similar findings have been reported indicating high levels of *Campylobacter* in cecal specimens (Luechtefeld et al, 1981; Cox et al, 2000), and detecting *Campylobacter* at levels as high as 10^7 CFU/g, which suggested that the ceca may be a favorable compartment of the intestinal tract for colonization.

The consumption of turkey is steadily rising. Currently, the human population is more health-conscious; therefore, they prefer to consume lean meat. Consequently, the consumption of turkey concurrent with the high prevalence of *Campylobacter* in turkey production results in relatively frequent incidences of human enteritis. *Campylobacter jejuni* infection was closely associated with the consumption of processed turkey, resulting in an outbreak in metropolitan Los Angeles (Shandera et al, 1992). Findings suggested that turkey carcasses maintained high levels of *Campylobacter*. Logue et al (2003) detected *Campylobacter* in turkey carcasses from two midwestern processing plants in the US. Both plants had relatively similar rates of *Campylobacter* contamination.

Other reports have indicated a high prevalence of *Campylobacter* in turkeys at processing (Luechtefeld and Wang, 1981; van Looveren et al, 2001). Although turkey products are processed, the microorganism may be present at substantial levels in retail turkey meat, thus, making the risk of human infection more probable. High levels of turkey carcass meat contamination was reported by Daczkowska-Kozon et al (1999), in which *Campylobacter* was isolated from 75% of turkey carcass samples and the number of campylobacters were highest in poultry (exceeding 10^3 CFU/cm^2) as compared to beef and pork. Conversely, Richter and Al-Sheddy (1990) described lower levels of microbial contamination in turkey meat as compared to other meat products, and *Campylobacter* was not detected in any of the turkey samples.

A series of events prior to or at slaughter could influence the prevalence of *Campylobacter* in turkeys, therefore, increasing the likelihood of contamination in retail meat. Wesley et al (2005) identified a significant difference in the prevalence of *Campylobacter* among turkey flocks, prior to transport and after the flocks reached the abattoir. Concurrently, shifts in species distribution were noted between sampling time points, with *C. coli* (17 profiles) having higher strain diversity than *C. jejuni* (seven profiles, J1 was most frequent) for both time points. Borck and Pedersen (2005) detected indistinguishable types between samples collected from the farm and at the time of slaughter (some were present throughout the process while others ceased to exist or were replaced), suggesting that strains have variable survival capabilities throughout processing.

1.9 *CAMPYLOBACTER* COLONIZATION IN SWINE

Although poultry are prominent carriers of *Campylobacter*, the organism is frequently isolated from the intestinal tract of swine (Oosterom et al, 1985; Harvey et al, 1999). Therefore, *Campylobacter* may naturally inhabit the gut flora and swine may potentially serve as vehicles of infection. Like turkeys, *Campylobacter* infection is asymptomatic in swine reservoirs. Such evidence has launched investigations to determine the prevalence of *Campylobacter* in the swine population and establish a correlation between swine and human campylobacteriosis.

Reports have indicated a high prevalence (70-100%) of *Campylobacter* isolated from swine in Canada (Guevremont et al, 2004), the US (Harvey et al, 1999), the Netherlands (Oosterom et al, 1985; Weijtens et al, 1993), Thailand (Padungtod and Kaneene, 2005), and Norway (Rosef et al, 1983). Conversely, a low level of prevalence was detected on a swine farm by Weijtens et al (2000). High levels of *Campylobacter* have been detected at different stages of production (Weijtens et al, 1993; Wesley et al, 1998), but it was likely that numbers declined as the swine aged (Weijtens et al, 1993). Wesley et al (1998) surveyed swine operations located in Iowa and North Carolina and isolated *Campylobacter* (90%, 93%, and 91%) from the nursery, grow-out, and finishing stages, respectively.

At the species level, swine are most frequently colonized by *C. coli*; thus, the organism may reside commensally within the animal host. Most studies have identified *C. coli* as the predominant species (Wesley et al, 1999; Kassa et al, 2005; Padungtod and Kaneene, 2005), hence, resulting in high carriage at time of slaughter (Pearce et al, 2003; Avrain et al, 2004). However, the organism may not be able to withstand the slaughter

process and can reach undetectable levels (Oosterom et al, 1985; Pearce et al, 2003). On the contrary, researchers have isolated *C. jejuni* from swine (Oosterom et al, 1985; Burch, 2002; Boes et al, 2005), albeit the frequency of recovery is not as high as that of *C. coli*. *Campylobacter jejuni* was identified in over 70% of healthy swine carriers in the Netherlands (Ooestrom et al, 1985). However, the numbers decreased significantly (to 9%) after slaughter, suggesting that high prevalence was possibly due to environmental sources within the slaughtering facility and not the intestinal tract being the original source of infection.

The nature of the rearing facility could potentially impact the prevalence of *Campylobacter* among swine herds. Extensive rearing allows the swine to free range, whereas, intensively reared systems confine the animals in a single area. Similar levels of *C. coli* prevalence (55%, 56%) were detected in swine produced in antibiotic-free extensive and intensive production systems, respectively (Gebreyes et al, 2005). However, the findings suggested that exposure to the outside environment may have contributed to the high prevalence in swine from extensively reared systems. The presence of additional farm animals may influence *C. jejuni* colonization in swine. Boes et al (2005) indicated no significant difference in the prevalence of *C. jejuni* among swine herds, with or without the presence of cattle or poultry. In this case, indistinguishable PFGE types were detected between herds of swine and cattle, which may be indicative of low-level transmission in mixed production systems (Boes et al, 2005).

Different approaches to a production scheme could result in a decline in herd contamination. The top-down approach was investigated to determine whether such an approach could result in *Campylobacter*-negative swine herds (Weijtens et al, 2000), in

which pathogen-free sows are supplied to top-breeding farms to produce piglets. *Campylobacter* was significantly higher in the breeding farms (98%) than in the pathogen-free sows (0%), but was detected at a very low level (14%) in breeding farms repopulated with pathogen-free sows. Thus, research findings suggested that the top down approach could help to achieve pathogen-free herds on known *Campylobacter*-positive farms (Weijtens et al, 2000), but for how long it can be maintained is unknown.

1.10 MODES OF TRANSMISSION

1.10.1 Introduction

Extensive research on *Salmonella* has launched current work on sources of poultry colonization by *Campylobacter* and other organisms associated with human enteritis (Newell and Fearnley, 2003), leading to observations in other meat animals (e.g. swine). A significant correlation between occurrence of *Campylobacter* and *Salmonella* infections in Danish broilers was not determined by Wedderkopp et al (2001). Both bacterial pathogens have been found to colonize broilers (Jacobs-Reitsma, 1995; Wilson, 2002; Cox et al, 2005), with *Campylobacter* being found more frequently. Likewise, the two organisms were also found to concurrently colonize the intestinal tract of swine in the Netherlands (Oosterom et al, 1985; Evans and Wegener, 2003). Notably, *Campylobacter*-positive and *Campylobacter*-free flocks have coexisted on the same farm (Stern et al, 2001), with slightly similar observations seen in swine (Alter et al, 2005), thus, making it difficult to understand the ecology of *Campylobacter*.

Campylobacter may infect animal populations through vertical and/or horizontal transmission. Studies have been conducted to validate these speculations in poultry (Lindblom et al, 1986; Chuma et al, 1994); however, no reports were found in regards to

swine. The issue of vertical transmission has been controversial, and the mechanism of *Campylobacter* being vertically transmitted from parent breeders to their progeny remains unclear. *Campylobacter* is capable of colonizing the reproductive system of mother breeders (Jacobs-Reitsma, 1994; Buhr et al, 2002; Hiett et al, 2002), and quite possibly can be passed on to the egg while in development. As for swine, *Campylobacter* was detected in about 58% of newborn piglets by Young et al (2000), suggesting direct transmission from infected sows. However, in a later study conducted in Germany *Campylobacter* was not detected in newborn piglets (Alter et al, 2005). The likelihood of vertical transmission was not considered in either study.

The temperature differential method (Clark and Bueschkens, 1985) and egg penetration studies (Doyle, 1984; Sahin et al, 2003) substantiate the invasive capabilities of *Campylobacter*; however, there were limitations to these tests. Experimentally, *C. jejuni* could be isolated from the egg surface or inner and outer membranes, but egg contents did not contain the organism.

Overall, there are few reports that indicate vertical transmission as a probable route, whereas many investigations have refuted vertical transmission (Acuff et al, 1982; Shanker et al, 1983; Doyle, 1984; van de Giessen et al, 1992; Sahin et al, 2003). Although the organism was present in high numbers among the parents (or breeders), eggs and newly hatched chicks were not colonized (Doyle, 1984; Sahin et al, 2003).

1.10.2 Horizontal Transmission

Horizontal transmission from the environment is the most probable source of *Campylobacter* infection in poultry (Sahin et al, 2002), as well as in swine (Young et al, 2000; Alter et al, 2005). Organic and free-range flocks have a higher prevalence of

Campylobacter in comparison to conventional flocks, presumably due to a higher level of environmental exposure (Heuer et al, 2001). Potential sources contributing to a horizontal mode of transmission may be water, improper cleaning and disinfection of houses, aerosols, human traffic, wild and domestic animals, flies and/or insects, and the presence of other farm animals being produced on the same farm (Newell and Fearnley, 2003). Farm exposure involves many different factors, therefore, these potential sources of infection must be identified in order to implement intervention strategies to mitigate and/or eliminate the occurrence of infection among meat animals.

1.10.3 Putative Sources of Horizontal Transmission

Feed and litter are relatively dry substances and such arid conditions may not accommodate microbial growth. Studies have indicated the inability to detect *Campylobacter* in feed and litter used in poultry and swine rearing (Kazwala et al, 1990; Humphrey et al, 1993; Jacobs-Reitsma et al, 1995; van de Giessen et al, 1998; Saleha, 2004; Alter et al, 2005), possibly due to its sensitivity to oxygen and drying. It was presumed by Montrose et al (1985) that *Campylobacter* could be transmitted between flocks via used litter, an assumption later proved to be erroneous (Payne et al, 1999). Overall, feed and litter do not appear to be critical to horizontal transmission of *Campylobacter* to turkeys or swine.

Water, especially untreated water, has been investigated as a source of flock infection (Kapperud et al, 1993; Pearson et al, 1993). *Campylobacter* has been detected in water samples obtained from poultry houses and swine barns (Saleha, 2004; Alter et al, 2005; Bull et al, 2006); however, water was not conclusively determined to be a source. An association was not determined by van de Giessen (1992), while others concluded that

water contamination typically occurred after the flock had become colonized (Engvall et al, 1986; Kazwala et al, 1990). Conflicting results have been obtained regarding water disinfection as an intervention strategy. Blaser et al (1986a) and Pearson et al (1993) reported positive effects of chlorination, whereas Stern et al (2002) concluded no effect with the use of chlorination.

Thorough cleaning and disinfection have been identified as means to improve biosecruity measures in order to reduce the incidence of *Campylobacter* infection in poultry and swine (van de Giessen, 1992; Evans and Sayer, 2000; Cardinale et al, 2004; Alter et al, 2005). Carryover is a probable event, thus, transmitting *Campylobacter* from one flock (or herd) to the next could be mediated by poorly managed rearing facilities. Inefficient cleaning of transport crates may also increase the risk of transmission (Ramabu et al, 2004; Hansson et al, 2005).

The role of aerosols in the spread of *Campylobacter* infection is poorly understood. Aerosols are desiccant substances that may be unfavorable for the growth of *Campylobacter*. It has been proposed that the ventilation system may influence transmission (Gibbons et al, 2001; Refregier-Petton et al, 2001); however, airborne transmission was excluded as a route of transmission by Pearson et al (1993). On the other hand, studies have reported isolating *Campylobacter* from air samples (Kazwala et al, 1990; Berndtson et al, 1996; Bull et al, 2006).

Animal production systems involve a lot of human trafficking by farm personnel for the sole purpose of routine husbandry practices. Frequent visits and rearing by multiple staff members have been associated with an increase in *Campylobacter* prevalence among animal populations (Refregier-Petton et al, 2001; Cardinale et al,

2004), suggesting that farm workers may facilitate the spread of *Campylobacter*. It is highly probable that workers may transfer *Campylobacter* from the outside environment via their work clothes, boots, or equipment. Many studies have indicated that the application of hygiene barriers significantly reduced the prevalence of *Campylobacter* among flocks (Kapperud et al, 1993; van de Giessen et al, 1996; Hald et al, 2000; Saleha, 2004). Biosecurity measures must be established and enforced by farm staff to minimize the incidence of *Campylobacter* infection.

The presence of vermin (Annan-Prah and Janc, 1988; Kapperud et al, 1993), wild birds (Pearson et al, 1993), and insects (Rosef and Kapperud, 1983; Shane et al, 1985; Jacobs-Reitsma et al, 1995; Gregory et al, 1997) have been associated with an increase in flock infection, and also confirmed through genotyping (van de Giessen et al, 1998; Hald et al, 2004b). Other studies have excluded them as putative sources of infection (Jacobs-Reitsma, 1992; Pearson et al, 1993; Evans and Sayers, 2000; Saleha, 2004). Alter et al (2005) isolated *C. coli* from a rodent and flies taken from the surrounding environment of swine barns, but it was not proven whether the environmental samples contained the same strains as the swine.

Campylobacter is widely distributed in nature, and can infect a wide range of animals grown for food. The presence of other animals on a farm may increase the risk of flock infection (Kapperud, 1994; van de Giessen et al, 1998; Cardinale et al, 2004); however, such an incident appears to occur infrequently in swine (Boes et al, 2005). It is a common practice for growers to produce multiple meat animals on a farm; therefore, strict division is a necessity to limit the spread of *Campylobacter* between animal hosts.

1.11 PATHOGENESIS OF *CAMPYLOBACTER*

1.11.1 Introduction

Although *C. jejuni* is the primary cause of enteritis in humans, there is relatively little knowledge of its mechanisms to cause human illness. Several virulence factors are involved in human gastroenteritis due to *Campylobacter* infection, but the underlying mechanisms are not adequately understood. The study of virulence factors associated with human gastroenteritis will enable researchers to form a better understanding of the pathogenesis of *Campylobacter*.

1.11.2 Motility

In order for *Campylobacter* to colonize the intestinal tract, organisms must be able to invade and move about the mucus layers. Szymanski et al (1995) investigated the role of motility as a means of cellular invasion to cause illness. *C. jejuni* and *C. coli* are highly motile due to the presence of a polar, unsheathed flagellum. The molecular weight of *Campylobacter* flagellin is 63,000 (Blaser et al, 1986b). In general, bacterial flagella consist of three parts: the filament, the hook, and the basal structure (Macnab and Aizawa, 1984).

It has been highly recognized that motility and the flagellum are pertinent virulence factors in the intestinal colonization of poultry. Nachamkin et al (1993) investigated the importance of a full intact flagellum for intestinal colonization. Motile wild-type strains were able to colonize three-day old chicks; however, flagellar mutants (nonmotile or partially motile) were unable to attain the same ability. Two genes, *flaA* and *flaB,* are responsible for expression of flagellin. The *flaA* gene is expressed at much higher levels than the *flaB* gene, and FlaA protein is the primary constituent in the

flagellum of *C. jejuni* (van Vliet and Ketley, 2001). Studies have shown the significance of both genes in regards to motility and invasion (Wassenaar et al, 1993; Wassenaar et al, 1994). On the contrary, another report indicated motility of *C. jejuni* mutants with inactivation of the *flaB* gene (Wassenaar et al, 1991). However, a defective *flaA* gene led to non-motile *C. jejuni* variants, with little potential to adhere to and penetrate into human intestinal cells (Wassenaar et al, 1991).

1.11.3 Chemotaxis

Chemotaxis is the ability of bacteria to detect and direct their movement through chemical gradients in the environment. Like motility, chemotaxis is an important factor in *Campylobacter* colonization of the avian intestinal tract. Khanna et al (2006) described the chemotactic ability of *C. jejuni* to be higher at a temperature of 37°C (human temperature) than at 42°C (avian temperature). A chemotactic response enables *Campylobacter* to direct their motion toward higher concentrations of attractants (e.g. food sources) and avoid repellents (e.g. poisons). Takata et al (1992) described the inability of nonchemotactic mutants of *C. jejuni* to colonize the intestinal tract of mice. The mutants failed to show any chemotactic behavior for any of the chemicals that acted as attractants for the wild-type strain.

The chemotactic response in *C. jejuni* is facilitated by a series of genes (*cheY* being the dominant RR), which enable the organism to monitor chemical changes within the environment and adjust accordingly. Marchant et al (1998) investigated the role of the *cheY* gene in the chemotatic ability of *C. jejuni*. Presumably, the rotational direction is influenced by an interaction between this particular protein and the flagellar motor. Temporal sensing allows *Campylobacter* to chemotax and "swim" in a straight line

before tumbling. However, tumbling occurs and a new direction is chosen if the bacterium is traveling in the wrong direction. *Campylobacter* tends to develop a different mechanism of motility in order to travel through environments with high viscosity (Ferrero and Lee, 1988). Likewise, *C. jejuni* was found to move along in a smooth pattern in a viscous medium, however, exhibited a darting motility at higher viscosities (Szymanski et al, 1995).

The chemotactic signal transduction pathway involves emitting signals to the flagella to reduce the chances of tumbling when the bacterium is crossing an area of high attractant concentration (Marchant et al, 2002). Mucin, a chemoattractant and the primary constituent of mucus, facilitates the growth of *C. jejuni* (Hugdahl et al, 1988). On the contrary, an area of high repellent concentration will increase the likelihood of tumbling in order for the bacterium to change direction. Hugdahl et al (1988) indicated that most constituents of bile were chemorepellents, except for the mucin component. Once the mucin component in bile is removed, bile exhibits a repellent effect, demonstrating the importance of soluble mucin in the chemoattraction of *C. jejuni* to bile (Hugdahl et al, 1988).

1.11.4 Adhesins and Invasion

Adhesins are molecules exposed on the bacterial surface that play an important role in the pathogenesis of *Campylobacter*. These structures facilitate the attachment of the pathogen to host cell receptors. *Campylobacter* may have several adhesins that work individually or together to promote adhesion to surface cells or binding to host receptors. Extensive research has been conducted on *in vitro* adherence in order to understand host-pathogen interactions involved in intestinal colonization and the protein-binding

mediators. However, there is insufficient knowledge of the host cell receptors that bind to *Campylobacter*. Studies of *C. jejuni* binding to INT 407 (human intestinal lining) and CaCo-2 (lining of human colon) cells have been conducted to mimic the actual host-pathogen interactions involved in *Campylobacter* colonization.

Presumably, INT 407 cells have receptors with specificity for *Campylobacter* adhesins, thus, enabling bacterial adhesion to these particular cells (McSweegan and Walker, 1986) *C. jejuni* exhibits enhanced binding and invasiveness in CaCo-2 cells (Szymanski et al, 1995). Mucus stimulates a chemotactic response in *C. jejuni* (Lee et al, 1986), and the ability of *C. jejuni* to colonize mucosal crypts is of importance in *Campylobacter* invasion (Lee et al, 1983; Lee et al, 1986; Szymanski et al, 1995).

Both the flagellum and lipopolysaccharide (LPS) of *C. jejuni* have been identified as adhesins. The absence of flagella reduced adherence to epithelial cells, thus motile variants of *C. jejuni* had greater adherence capabilities than did non-motile variants (McSweegan and Walker, 1986). Flagellated strains were able to adhere to INT 407 cells, as well as to the intestinal mucus. LPS was able to bind to both the epithelial cells and to intestinal mucus (McSweegan and Walker, 1986). The data indicated binding specificity between LPS and INT 407 cells and surface protein interaction with LPS.

Outer membrane proteins (OMPs) have been identified and associated with the binding and invasion of *Campylobacter* to host cells (DeMelo and Pechere, 1990). Two proteins, PEB1 and CadF, have been identified as mediators of *C. jejuni* binding to epithelial cells and fibronectin, respectively. Pei et al (1998) described the role of PEB1 in bacterial adhesion. Inactivation of *peb1A*, that encodes PEB1, resulted in a significant reduction in adherence of *C. jejuni* to epithelial cells. In Gram-negative bacteria, binding

components of ABC transport systems are located in the periplasmic space, a location that inhibits interaction between the binding components and epithelial cells (Pei et al, 1998). However, PEB1 is located on the surface of *C. jejuni*, thus, substantiating its role in adherence.

CadF, the 37-kDa OMP encoded by *cadF* genes in *Campylobacter* isolates, mediates the binding of *C. jejuni* to fibronectin (Konkel et al, 1997). Konkel et al (1999) indicated that the CadF protein was conserved among all of their tested *C. jejuni and C. coli* isolates, and the *cadF* gene was amplified in more than 90% of the isolates. Experimental animal models were utilized to affirm the role of adhesins in cell invasion. Pei et al (1998) compared mouse intestinal colonization of a *C. jejuni* mutant, without the PEB1 protein, to the wild type strain. The mutant strain exhibited a decreased ability to adhere to and invade epithelial cells (Pei et al, 1998). The *cadF* mutant was not recovered from newly hatched chicks, therefore, validating the importance of CadF protein in the colonization of *C. jejuni* (Ziprin et al, 1999). Another study described reductions in fibronectin binding of mutants due to disruptions of the *cadF* gene (Konkel et al, 1997).

1.11.5 Toxins

Research has indicated that the toxigenic nature of *Campylobacter* plays an integral role in the pathogenesis of the organism resulting in diarrheal illness. Enterotoxins and cytotoxins are the two types of toxins involved in human enteritis. Enterotoxins bind to host cell receptors, gain entry into the cell, and elevate intracellular cAMP levels, whereas cytotoxins kill target cells via intracellular activity or pore formation in cells (Wassenaar, 1997).

Ruiz-Palacios et al (1983) were the first to document the enterotoxigenic activity of *C. jejuni*. The elongation of CHO cells and rounding of Y-1 cells *in* vitro signify enterotoxin activity of *C. jejuni* culture supernatants (Wassenaar, 1997). Bacteria approaching stationary phase were found to have maximum toxigenic activity (Klipstein and Engert, 1984), and culture conditions were also influential factors in toxin yields. The presence of iron in culture medium may be a necessity for elevated toxin production. McCardell et al (1986) concluded that iron influenced enterotoxin production, and the addition of ferric chloride greatly increased toxin yields. Cytolethal distending toxin (CDT)-positive strains were grown in deferrated medium, which resulted in lack of toxin or extremely low toxin production.

CDT was first described by Johnson and Lior (1988), due to its progressive cell distention and cytotoxigenic effect on sensitive cells. The study reported that CDT was effective against CHO, Vero, HeLa, and Hep-2 cells, but Y-1 cells were not affected. The cytotoxigenic effect is mediated by the three genes, *cdtA, cdtB, and cdtC*, located in tandem. The molecular weights of the three proteins are 30, 28, and 21 kDa, respectively (Pickett et al, 1996). Pickett et al (1996) conducted the first study to isolate and characterize toxin genes of *Campylobacter*. They were able to analyze the three structural genes involved in cytotoxin production via cloning and sequencing, and concluded that the genes can be detected in a majority of *Campylobacter* strains. However, at the species level gene expression to elicit toxin activity was variable (Pickett et al, 1996).

1.11.5.1 Incidence of Enterotoxin and Cytotoxin Production

Studies have investigated the prevalence of enterotoxin and cytotoxin production in conjunction with human diarrheal illness. Wassenaar (1997) indicated the relative occurrence of enterotoxin production of *Campylobacter* from different regions of the world. The frequency of enterotoxin production may be species-specific, with reports indicating that enterotoxigenic activity occurred more frequently in *C. jejuni* than *C. coli* (Belbouri and Megraud, 1988; Lindholm et al, 1990).

Enterotoxigenic activity has been closely associated with watery diarrhea, whereas, cytotoxigenic activity results in inflammatory and bloody diarrhea (Walker et al, 1986). Florin and Antillon (1992) isolated *C. jejuni* strains that elicited an enterotoxic effect more frequently from Costa Rican children with watery diarrhea than those with bloody or inflammatory diarrhea. Similar findings indicated that enterotoxigenic *C. jejuni* more readily resulted in watery diarrhea, and colonization of non-enterotoxigenic strains led to asymptomatic infections in carriers (Ruiz-Palacios et al, 1983).

Cytotoxin production has been investigated and associated with a higher level of severe diarrheal illness. Klipstein et al (1985) reported a correlation between the pathogenic properties of infective *C. jejuni* and clinical manifestations in an infected host. All cytotoxin-positive strains were isolated from patients with bloody invasive-type diarrhea, whereas, watery secretory-type diarrhea was noted in patients with enterotoxin-positive strains. The study conducted by Florin and Antillon (1992) indicated that patients experienced either watery or inflammatory diarrheal illness. However, cytotoxin production was detected in strains isolated from patients with inflammatory diarrhea.

Other studies have investigated the major proteins that mediate the invasion and

cytotoxic activity of *Campylobacter*. Lara-Tejero and Galan (2001) described the combination of the three CDT polypeptides (cdtABC) as an active tripartite holotoxin. The role of the *cdtB* gene is to damage host cell DNA, which triggers cell cycle arrest. The genes, *cdtA* and *cdtC*, serves as delivery vehicles of *cdtB* into the host cell. The triad of genes has been detected in isolates of human and animal origin (Eyigor et al, 1999; Bang et al, 2003; AbuOun et al, 2005; Rozynek et al, 2005). Speculations arise concerning the role of CDT in human enteritis, when clinical isolates are CDT-negative even though the patients have diarrheal symptoms. AbuOun et al (2005) suggested that mutations, due to deletions or changes in nucleotide sequence, might account for toxin negativity. However, it was recognized that serum from infected patients neutralized the cytotoxin, thus, indicating that such activity is probable during human infection (AbuOun et al, 2005).

1.11.6 Iron Acquisition

Iron is a necessity in the pathogenesis of *Campylobacter*. Sufficient iron acquisition for essential metabolic roles is required for the establishment of *C. jejuni* infection (Kelly, 2001). As a pathogen, *C. jejuni* is likely to compete with the host and normal microbiota for iron sources (Holmes et al, 2005). Iron withholding is a known host defense mechanism against an invading pathogen (Ratledge and Dover, 2000); however, these pathogens have developed mechanisms to seek out iron sources to assist in colonization and survival within the host (Konkel et al, 2001). The availability of free iron in mammalian hosts is inadequate to facilitate the growth of *Campylobacter*. Iron is contained within the host cells as haem or in ferritin and bound to transferrin (in blood) and lactoferrin (mucous layers).

In response to an iron-deficient environment, *Campylobacter* may produce siderophores to acquire iron from the host. Field et al (1986) indicated that not all strains of *C. jejuni* are able to produce siderophores. However, *C. jejuni* can also acquire iron by using siderophores (e.g. enterochelin and ferrichrome) produced by other microorganisms. Siderophore-mediated iron uptake systems are important in the pathogenesis of bacterial infections (Richardson and Park, 1995). The synthesis of siderophores and corresponding uptake systems is often iron-repressed (Konkel et al, 2001).

There are several siderophore-mediated systems involved in iron acquisition of *C. jejuni*. The utilization of enterochelin as an iron source is mediated by the genes *ceuBCDE*, which encode this transport system (Richardson and Park, 1995). Defective mutants were unable to utilize the siderophore as a source of iron. Guerry et al (1997) identified two genes involved in iron uptake of *Campylobacter* strains. The *cfraA* gene encoded an iron-repressible outer membrane protein, whereas, the TonB-like protein was involved in the utilization of hemin, ferrichrome, and enterochelin. Galindo et al (2001) identified the genes, *cfhuABD*, involved in the ferrichrome uptake system. Limiting the availability of iron induced the expression of the 80-kDa OMP by *C. jejuni*, likely involved in ferrichrome uptake (Galindo et al, 2001). A haemin uptake system encoded by the genes *chuABCD* has been identified as being involved in iron acquisition (Rock et al, 1999). Pickett et al (1992) described the ability of hemin and hemoglobin to stimulate growth of *C. jejuni* strains under low iron conditions. The researchers detected the inability of mutant strains to utilize these iron sources concomitant to the absence of an iron-regulated 71-kDa OMP in some of the mutants. This particular protein was present

in the wild-type strain, thus, indicating the significance of the protein in iron acquisition (Pickett et al, 1992).

Typically, ferric iron acquisition systems in Gram-negative bacteria, such as *Campylobacter*, will consist of an outer membrane receptor (transports an iron compound across the outer membrane), a periplasmic binding protein, and an inner membrane ABC transporter consisting of a permease and ATP-binding protein (van Vliet and Ketley, 2001). The TonB protein is essential in energy transduction for several iron uptake systems, and the genes ExbBD may facilitate the functionality of the protein (Kelly, 2001). A TonB-like protein detected in *Campylobacter* was necessary for the utilization of different iron sources (Guerry et al, 1997); however, a defect in the gene encoding the protein resulted in the inability of *Campylobacter* to utilize iron sources.

The *C. jejuni* genome encodes a putative ferrous iron uptake system (FeoB protein) (van Vliet and Ketley, 2001). The product of the ferrous iron uptake gene, *feoB*, in *C. jejuni* shares 29% and 50% amino acid identity to ferrous iron uptake proteins in *E. coli* and *H. pylori*, respectively (Raphael and Joens, 2003). There is little knowledge of the role of the FeoB protein in *Campylobacter* infection. However, reports have indicated that *feoB* mutants from other enteric pathogens were unable to colonize the intestinal tract (Stojiljkovic et al, 1993; Velayudhan et al, 2000). Conversely, an investigation conducted by Raphael and Joens (2003) indicated that FeoB was not a requirement for iron acquisition in *C. jejuni* because gene mutations did not affect ferrous iron uptake.

Fur (ferric uptake regulator) homologs are ubiquitous, metal-responsive regulatory proteins, involved in the regulation of metal homeostasis, oxidative stress

defense, and acid resistance (van Vliet et al, 2002). The Fur protein, encoded by the *fur* gene, represses the transcription of iron-regulated promoters in response to an increasing intracellular iron concentration (van Vliet et al, 1998). Studies have investigated the role of Fur in iron acquisition and how the protein regulates the expression of genes involved in the iron-responsive regulatory system (van Vliet et al, 1998; Palyada et al, 2004; Holmes et al, 2005).

Holmes et al (2005) indicated that iron limitation resulted in the expression of siderophore-mediated systems at higher levels in the wild-type strain than in the *fur* mutant. However, higher levels of expression were detected in the *fur* mutant under iron-rich conditions, suggesting that the transport systems and genes involved in iron uptake were Fur-regulated (Holmes et al, 2005). van Vliet et al (1998) reported a slower rate of growth in the *fur* mutant than in the wild-type under both iron-limited and iron-rich conditions, and also detected several genes whose expression was iron-regulated in the parental strain, but not in the mutant. Palyada et al (2004) detected a significantly reduced rate of colonization of the *fur* mutant in comparison to the wild-type strain in two-day old chicks, thus, indicating the significance of iron homeostasis *in vivo*.

1.12 ANTIBIOTIC RESISTANCE

1.12.1 Introduction

Campylobacter is the leading causative agent of acute diarrheal disease in human in the United States. Gastroenteritis-causing pathogens are the second leading cause of morbidity and mortality worldwide, with *Salmonella*, *Shigella*, and *Campylobacter* being the principal species accounting for up to 20% of acute diarrhea observed worldwide (Streit et al, 2006). Most enteric cases due to *Campylobacter* infection are self-limiting;

thus, further medical attention is not sought after. However, instances of severe or recurrent illness would require antibiotic treatment. Several different antibiotics have been administered to treat acute gastroenteritis, preferably macrolides, fluoroquinolones (FQ), and tetracyclines. Bacterial resistance will adversely impact the effectiveness of antimicrobial agents; therefore, they must be used carefully in human and veterinary practice.

Macrolides are the antimicrobial agents of choice in treating *Campylobacter* infections. Streit et al (2006) reported macrolides as highly potent agents against *Campylobacter* spp. in clinical cases of gastroenteritis from Europe and Latin America, with erythromycin displaying a susceptibility rate of 91%. Engberg et al (2001) summarized various reports from around the world, indicating a higher frequency of erythromycin resistance in *C. coli* (0 to 68%) than *C. jejuni* (0 to 11%). Erythromycin-resistant strains have little prevalence in some countries (Itoh et al, 1995; Dowling et al, 1998; Gaudreau and Gilbert, 1998; Varga and Fodor, 1998) than in others, which have developed resistance (Lim and Tay, 1992; Hoge et al, 1998; Li et al, 1998).

Fluoroquinolones (FQ), particularly ciprofloxacin, have been used to treat enteritis and as a means to prevent travelers' disease. A significant increase in the incidence of ciprofloxacin-resistant *Campylobacter* isolates has been reported worldwide (Rautelin et al, 1991; Chatzipanagiotou et al, 1993; Li et al, 1998; Saenz et al, 2000; Nachamkin et al, 2002; Luber et al, 2003; Feierl et al, 1994; Gupta et al, 2004). Numerous countries have reported an association between foreign travel and quinolone-resistant *Campylobacter* (Sjogren et al, 1997; Smith et al, 1999; Afset and Maeland,

2001; The *Campylobacter* Sentinel Surveillance Scheme Collaborators, 2002; Hakanen et al, 2003; Unicomb et al, 2003; Engberg et al, 2004).

Tetracyclines are a third well-known class of antibiotics used to treat human gastroenteritis. Many studies have reported a high frequency of tetracycline resistance in *Campylobacter* isolates (Lee et al, 1994; Gaudreau and Gilbert, 1998; Li et al, 1998; Ge et al, 2003; Desmonts et al, 2004; Gibreel et al, 2004; Pratt and Korolik, 2005; Luangtongkum et al, 2006). *C. coli* isolates were found to be frequently more resistant to tetracycline than *C. jejuni* isolates in studies conducted by Luber et al (2003) and Desmonts et al (2004). Conversely, Li et al (1998) and Ge et al (2003) indicated a higher level of tetracycline resistance in *C. jejuni* isolates (95%, 81%) than *C. coli* (85%, 77%), respectively.

1.12.2 Controversy with Antibiotic Use in Agriculture

Antibiotic use in food animal production is of great concern from a food safety and public health perspective. Antibiotics are compounds with antimicrobial activity that can be administered orally, parenterally or topically-are used in human and veterinary medicine to treat and prevent disease, and for other purposes including growth promotion in food animals (Phillips et al, 2004). Typically, antibiotics are used appropriately as therapeutic agents against bacterial infections, but there is potential misuse in both human medicine and agriculture (Khachatourians, 1998).

The use (and/or misuse) of antibiotics in animals and humans results in the emergence and dissemination of resistant bacteria (Anderson et al, 2003). Resistant bacteria find passage through the food supply to the human population, resulting in resistant infections (Anderson et al, 2003). These bacterial strains that have acquired

antibiotic resistance will lessen the efficacy of antibiotics used for human treatment, thus, signifying pertinent public health implications. Although therapeutic use of antibiotics in animal production promotes animal health and provides a sufficient supply of meat and other animal products, the preservation of long-term effectiveness of antibiotics used in human medicine is vital (Anderson et al, 2003).

Since the 1950's antibiotics have been used in livestock production to treat infections and improve growth and feed efficiency (Anderson et al, 2003). The extensive use of antibiotics given to food animals is for sub-therapeutic purposes (e.g. growth promotion). Growth promoter use creates a major food animal reservoir of resistant bacteria, with a potential for spread to humans by food intake or by animal contact (Wegener, 2003). Khachatourians (1998) indicated that about 90% of the antibiotics used in agriculture are given as growth-promoting and prophylactic agents, rather than to treat infection. Such agents have been banned in Europe (Evans and Wegener, 2003). Numerous classes of antibiotics have been approved for growth promotion and feed efficiency in the United States, including tetracyclines, penicillins, and macrolides (Anderson et al, 2003).

1.12.3 Epidemiology

1.12.3.1 Humans

The emergence of resistant bacterial strains abates the efficacy of antibiotics used to treat clinical cases of campylobacteriosis. Resistant strains will affect how the human body responds to a *Campylobacter* infection. The incidence of antibiotic-resistant human clinical isolates has been reported in different countries (Wang et al, 1984; Lim and Tay, 1992; Gaunt and Piddock, 1996; Thwaites and Frost, 1999; Moore et al, 2001; Luber et

al, 2003; Uzunovic-Kamberovic, 2003; Engberg et al, 2004; Dingle et al, 2005; Pratt and Korolik, 2005).

The increase in FQ resistance in *Campylobacter* isolates of human origin is closely associated with the clinical use and/or overuse of this class of antibiotics in animal production systems (Li et al, 1998; Thwaites and Frost, 1999; Saenz et al, 2000; Bae et al, 2005; Humphrey et al, 2005; Luangtongkum et al, 2006). Many countries have reported marked increases in FQ resistance in human isolates (Feierl et al, 1994; Hoge et al, 1998; Smith et al, 1999; Desmonts et al, 2004). These findings signify the public health implications regarding the overuse of antibiotics in animals intended for human consumption.

1.12.3.2 Animal Livestock

Excessive use of antibiotics in animal production has led to resistant bacteria in the food supply. Growth promotion and feed efficiency are the primary reasons for the use of antibiotics in food animals. Typically, animals are asymptomatic carriers of enteropathogens, such as *Campylobacter*. *Campylobacter* infections are not severe in animal reservoirs; therefore, animals remain intestinal carriers up to slaughter. Due to public outcry, some countries banned the use of antibiotics in livestock agriculture resulting in the declination of antibiotic-resistant strains of animal and human origin (Bager et al, 1999; Klare et al, 1999; Pantosti et al, 1999; van den Bogaard et al, 2000; Aarestrup et al, 2001; Bager and Emborg, 2001; Wegener, 2003)

1.12.3.3 Turkeys

Incidences of high levels of antibiotic resistance in turkeys have been reported (Ge et al, 2003; Luber et al, 2003; Luangtongkum et al, 2006). Ge et al (2003) and

Luangtongkum et al (2006) indicated high frequencies of antibiotic resistance to tetracycline, erythromycin, and fluoroquinolones in *Campylobacter* isolates from turkeys. *Campylobacter* isolates from turkeys have also shown various levels of resistance to other classes of antibiotics such as lincosamides, aminoglycosides, and beta lactams (Ge et al, 2003; Luber et al, 2003; Luangtongkum et al, 2006). The species of the organism and the source of isolation (e.g. turkeys vs. humans) are probable causes of varying resistance rates among *Campylobacter* isolates (Luber et al, 2003).

Markedly, high levels of resistance to ciprofloxacin and erythromycin have been observed in turkeys (Ge et al, 2003; Luangtongkum et al, 2006). Coresistance to ciprofloxacin and erythromycin must be considered highly undesirable, as the two antibiotics are generally advocated as first-line drugs to treat campylobacteriosis (Ge et al, 2003). Multidrug resistance has been observed among *Campylobacter* isolates from conventionally raised turkey flocks (Luangtongkum et al, 2006), with the inclusion of antibiotic usage in conventional production influencing the prevalence of antibiotic-resistant *Campylobacter* (Luangtongkum et al, 2006).

1.12.3.4 Swine

Numerous studies have investigated antibiotic resistance in *Campylobacter* isolates from swine (Aarestrup et al, 1997; Saenz et al, 2000; van Looveren et al, 2001; Evans and Wegener, 2003; Avrain et al, 2004; Gebreyes et al, 2005; Thakur and Gebreyes, 2005), also indicating resistance to previously discussed first-line antibiotics used for human treatment of *Campylobacter* infection. In particular, a high frequency of erythromycin resistance has been reported (Wang et al, 1984; Elharrif et al, 1985; Aarestrup et al, 1997; Saenz et al, 2000; van Looveren et al, 2001), with MICs of 32

μg/ml (Wang et al, 1984) and greater than 128 μg/ml (Elharrif et al, 1985) being detected among *C. coli* isolates from swine. Resistance to clindamycin, ampicillin, streptomycin, and gentamicin has also been reported (Wang et al, 1984; Elharrif et al, 1985; Aarestrup et al, 1997; Saenz et al, 2000; van Looveren et al, 2001; Avrain et al, 2004).

Like poultry studies, research has been conducted to determine how the type of production system in which the herds are produced may impact the prevalence of antibiotic-resistant *Campylobacter* from swine. Extensive and intensive systems were investigated by Gebreyes et al (2005), at which antibiotics were not given for therapeutic purposes or as growth promoters. However, isolates were highly resistant to tetracycline (48.6%) and erythromycin (39.7%), and showed resistance to ciprofloxacin (which is not licensed for use in any U.S. swine production system) (Gebreyes et al, 2005). Saenz et al (2000) also reported a high level of ciprofloxacin resistance (100%) in *C. coli* strains from swine.

1.13 ANTIBIOTIC RESISTANCE MECHANISMS

1.13.1 Introduction

Like any other bacteria, *Campylobacter* species develop resistance mechanisms, which abate their susceptibility to different antibiotics. Resistance to an antibiotic can be either intrinsic or acquired, in which all strains within a species can be resistant to an antibiotic or strains acquire resistance due to a chromosomal mutation or uptake of foreign DNA (e.g. plasmid or transposon) (Taylor and Courvalin, 1988). There are a number of antibiotics to which all *C. jejuni* and *C. coli* isolates are intrinsically resistant; however, the mechanisms are unknown (Taylor and Courvalin, 1988).

1.13.2 Tetracycline Resistance

Taylor et al (1980) indicated that tetracycline resistance in *C. jejuni* was shown to be plasmid-mediated. Taylor (1986) and Ng et al (1987) reported plasmids ranging in size of 40 to 50 kb, whereas other studies have indicated larger size ranges of 2 to 162 kb and 16 to 208 kb, respectively (Tenover et al, 1985; Lee et al, 1994). Several countries have reported plasmids encoding tetracycline resistance in *Campylobacter* strains of different origins (Taylor et al, 1983; Tenover et al, 1983; Ng et al, 1987; Lee et al, 1994; Gibreel et al, 2004; Pratt and Korolik, 2005).

Tenover et al (1983) described two plasmids (pFKT1000 and pFKT2000) detected in *C. jejuni* strains screened for plasmid-mediated tetracycline resistance, and found that resistant clones harbored both plasmids; however, susceptible clones only harbored one of the plasmids. Taylor et al (1983) characterized several plasmids responsible for tetracycline resistance in *Campylobacter* strains, and detected high levels of tetracycline resistance ($\geq$64 μg/ml) among the closely related strains.

The *tet*(O) gene mediates tetracycline resistance in one of four ways: efflux of tetracycline, modification of tetracycline, ribosomal protection, or mutation of the 16S rRNA (Trieber and Taylor, 2000). Tet(O) protein acts as a ribosomal protection protein that confers resistance by displacing tetracycline from its primary binding site on the ribosome (Taylor and Courvalin, 1988). Tetracycline resistance in tested isolates has been found to be mediated through the *tet*(O) gene (Gibreel et al, 2004; Thakur and Gebreyes, 2005). DNA probes (*tetO, tetM*) have been used to detect and locate tetracycline resistance in *Campylobacter* isolates from poultry and other sources (Ng et al, 1987; Lee et al, 1994).

1.13.3 Aminoglycoside Resistance

Resistance to aminoglycosides (e.g. kanamycin and streptomycin) in *Campylobacter* strains of clinical and animal origin has been reported (Ouellette et al, 1987; Sagara et al, 1987; Cabrita et al, 1992; Aarestrup et al, 1997; Schuppers et al, 2005; Luangtongkum et al, 2006). Kanamycin and streptomycin are protein synthesis inhibitors, interfering with A-site binding and causing errors in translation (Trieber and Taylor, 2000). The antibiotic is modified and lacks the ability to interact with the ribosome, resulting in resistance. Aminoglycoside phosphotransferases, aminoglycoside adenylyltransferases, and acetyltransferases are the enzymes responsible for resistance to aminoglycosides (Trieber and Taylor, 2000).

Most reports indicate plasmid-mediated resistance to aminoglycosides (Kotarski et al, 1986; Sagara et al, 1987; Papadopoulou and Courvalin, 1988; Tenover et al, 1992); however, resistance can be chromosomally encoded (Ouellette et al, 1987; Taylor et al, 1988). Ouellette et al (1987) determined that APH(3')-I, a resistance determinant mediated kanamycin resistance in BM2196, a *Campylobacter*-like clinical strain.

1.13.3.1 Kanamycin

Lambert et al (1985) and Rivera et al (1986) were the first to describe kanamycin resistance in *Campylobacter*. Kanamycin resistance in *C. coli* strain BM2509 was due to synthesis of a 3'-aminoglycoside phosphotransferase of type III (APH(3')-III), an enzyme confined to Gram-positive cocci (Lambert et al, 1985). Rivera et al (1986) indicated that kanamycin resistance in *C. coli* strain 981 was mediated by phosphotransferases APH(3') type-IV and APH(3"). None of the plasmids in either study were transferable to *E. coli* by conjugation.

Taylor et al (1988) used the restriction map of plasmid pIP1433 from strain BM2509 to locate the kanamycin resistance marker in a *C. coli* strain (U4696) using an *aphA*-3 probe. The resistance determinant was located on the chromosome and specified a 3'-aminoglycoside phosphotransferase of type III, as seen with plasmid pIP1433 in strain BM2509 (Taylor et al, 1988). A cloning experiment conducted by Trieu-Cuot et al (1985) concluded that the *aphA*-3 gene from *C. coli* plasmid pIP1433 conferred kanamycin resistance in *E. coli*.

1.13.3.2 Streptomycin

Campylobacter coli strains BM2509 (Lambert et al, 1985) and 981 (Rivera et al, 1986), as well as CLO BM2196 (Ouellette et al, 1987) were all streptomycin-resistant. Production of a 6-aminoglycoside adenylyltransferase accounted for streptomycin resistance in BM2509 (Lambert et al, 1985). Streptomycin resistance in CLO strain BM2196 was mediated through a 3"-9-aminoglycoside adenylyltransferase, with resistance being chromosomally encoded (Ouellette et al, 1987).

Streptomycin and/or spectinomycin-resistant *Campylobacter* strains were studied by Pinto-Alphandary et al (1990), due to the modifying enzymes APH(3"), APH(6), and AAD(3")(9). All tested strains had adenylylating activity with the *aadE* gene being found most frequently among the strains (Pinto-Alphandary et al, 1990), and levels of streptomycin resistance correlated with adenylyltransferase production effective against streptomycin.

1.13.4 Macrolide Resistance

Macrolides (e.g. erythromycin, azithromycin) are protein synthesis inhibitors that bind to the 23S rRNA of the 50S ribosome unit, thus, creating resistance through

modification of the target site or through alteration of the antibiotic (Trieber and Taylor, 2000). Erythromycin binds to the ribosome and causes dissociation of the peptidyl-tRNA rather than blocking peptidyltransferase activity (Trieber and Taylor, 2000). *In vivo* protein synthesis in erythromycin-susceptible *Campylobacter* strains was inhibited by low levels of erythromycin (5 μg/ml), whereas high concentrations (100 μg/ml) had no effect on protein synthesis in erythromycin-resistant strains (Yan and Taylor, 1991). Unlike tetracycline, erythromycin resistance in *Campylobacter* species is not plasmid-mediated but quite possibly chromosomally encoded (Yan and Taylor, 1991).

Reports have indicated that erythromycin resistance in *Campylobacter* is encoded by a point mutation in the 23S rRNA gene (Jensen and Aarestrup, 2001; Vacher et al, 2003; Thakur and Gebreyes, 2005; Kim et al, 2006). High erythromycin MICs (128 to >1024 μg/ml) have been detected in *Campylobacter* isolates in which point mutations were identified (Niwa et al, 2001; Payot et al, 2004; Gibreel et al, 2005).

Transformation studies involving macrolide resistance have been conducted to determine whether the resistance phenotype can be transferred to susceptible isolates (Gibreel et al, 2005; Kim et al, 2006). Kim et al (2006) concluded that transformation to erythromycin resistance was more frequent in turkey strains than swine. Erythromycin resistance was found to be stable after repeated subcultures without the selective pressure (Gibreel et al, 2005; Kim et al, 2006).

1.13.5 Fluoroquinolone Resistance

Quinolones have been available in human medicine since the mid-1960s, and the first fluoroquinolone (FQ) (ciprofloxacin) was approved for use in humans in 1986, followed by licensing of veterinary use in poultry in the mid-1990s (Gupta et al, 2004).

Due to the association of FQ use in agriculture and the emergence of resistance in humans, FDA proposed to withdraw FQ use in poultry in 2000 (Gupta et al, 2004). Consequently in July 2005, the use of FQs was banned (http://www.farmedanimal.net/faw/faw5-34.htm).

Studies have proposed an association between the agricultural use of FQs and the increase in FQ-resistant *Campylobacter* strains (Endtz et al, 1991; Reina et al, 1992; Sanchez et al, 1994; van Looveren et al, 2001). It has been found that FQ treatment influences the prevalence and persistence of resistant strains among poultry flocks (McDermott et al, 2002; Humphrey et al, 2005), in which treatment resulted in the rapid development of resistance. Research findings have indicated that clinical strains of *Campylobacter* were more susceptible to FQs than those of animal origin (Wang et al, 1984; Li et al, 1998; Saenz et al, 2000; Luber et al, 2003).

Nalidixic acid, a quinolone, has been used for FQ resistance screening due to the close correlation between quinolone and FQ resistance among *Campylobacter* strains (Gupta et al, 2004). Cross-resistance between nalidixic acid and ciprofloxacin in *Campylobacter* strains has been described (Smith et al, 1999; Schuppers et al, 2005; Luangtongkum et al, 2006), as well as in resistant mutants (Taylor et al, 1985; Gootz and Martin, 1991).

The mechanisms of FQ resistance are alterations in drug target (DNA gyrase, topoisomerase IV), alterations in drug permeability, and efflux (Trieber and Taylor, 2000). FQs are DNA synthesis inhibitors through interaction with complexes composed of DNA and either DNA gyrase or topoisomerase IV (Hooper, 2001), both are essential for DNA replication, recombination, and transcription (Trieber and Taylor, 2000). The

DNA gyrase subunits, GyrA and GyrB, are homologous with ParC and ParE subunits of topoisomerase IV, respectively (Hooper, 2001).

Alterations in target enzymes (localized to specific domains of each subunit type) have been the most extensively studied mechanism of FQ resistance, as a result of chromosomal mutations in genes encoding subunits of DNA gyrase and topoisomerase IV (target enzymes) and those affecting the expression of efflux systems (Hooper, 2001). The latter being less frequently reported. The most common mutation that occurs resulting in FQ resistance involves the DNA gyrase subunit A (*gyrA*) gene. Gootz and Martin (1991) determined that DNA gyrases from the resistant mutants were less susceptible than the wild-type enzyme to inhibition by FQs, and an alteration in the DNA gyrase subunit A was the cause of high level resistance.

Resistance of *C. jejuni* and *C. coli* to both old and new FQs mainly depends on mutations in the Quinolone Resistance Determining Region (QRDR) in the *gyrA* encoding DNA gyrase subunit A (Niwa et al, 2003). Several different molecular analyses have been utilized to characterize the QRDR of *gyrA* in quinolone-resistant *Campylobacter* isolates. Direct sequencing has been the primary approach (Husmann et al, 1997; Gibreel et al, 1998; Cooper et al, 2002; Luo et al, 2003; Alonso et al, 2004; Corcoran et al, 2005; Jesse et al, 2006).

Other methods found to be successful in characterizing the QRDR of *gyrA* include single-strand conformation polymorphism (SSCP) (Charvalos et al, 1996; Hakanen et al, 2002; Piddock et al, 2003; Beckmann et al, 2004), mismatch amplification mutation assay (MAMA-PCR) (Zirnstein et al, 1999; Zirnstein et al, 2000; Lucey et al, 2002; Payot et al, 2002), fluorogenic PCR assay (Wilson et al, 2000; Padungtod et al, 2003).

Additional methods that have been used were the microelectronic chip assay, quinolone line probe assay, and PCR-restriction fragment length polymorphism (Westin et al, 2001; Niwa et al, 2003; Alonso et al, 2004).

The Thr-86-Ile point mutation has been the most frequently reported single nucleotide change in FQ-resistant *Campylobacter* strains, a mutation in codon 86 resulting in a threonine-to-isoleucine substitution. Wilson et al (2000) indicated that the predominance of the C to T transition in codon 86 of *gyrA* among highly FQ-resistant *Campylobacter* strains is indicative of the functional role of *gyrA* in conferring high level FQ resistance. Elevated levels of FQ resistance (or high MICs to FQs, as reported in each individual study) observed in *Campylobacter* strains correlates with detection of the Thr-86-Ile mutation, which has also been described in mutant strains (Gootz and Martin, 1991; Wang et al, 1993; Ge et al, 2005). However, other studies were unable to make a correlation (Corcoran et al, 2005) or have reported FQ resistance in the absence of *gyrA* mutations (Lucey et al, 2002; Ge et al, 2003; Oza et al, 2003; Piddock et al, 2003; Chuma et al, 2004; Jesse et al, 2006).

Additional point mutations found in *Campylobacter* isolates of clinical and animal origin, as well as laboratory-derived mutants, have been reported (Wang et al, 1993; Charvalos et al, 1996; Ruiz et al, 1998; Zirnstein et al, 1999; Bachoual et al, 2001; Luo et al, 2003; Chu et al, 2004; Chuma et al, 2004; Corcoran et al, 2005; Jesse et al, 2006). Studies have also reported double mutations resulting in FQ resistance (Hakanen et al, 2002; Payot et al, 2002; Ge et al, 2003; Piddock et al, 2003; Beckmann et al, 2004; McIver et al, 2004; Ge et al, 2005).

Attempts have been made to identify mutations in the *gyrB* and *parC* genes, which are also targets of FQs. Sequencing analysis revealed no mutation in the QRDR of *gyrB* in *Campylobacter* isolates (Bachoual et al, 2001; Cooper et al, 2002; Payot et al, 2002). However, Piddock et al (2003) detected a change in *gyrB* in 4% of *Campylobacter* isolates, but the alterations were related to silent mutations in gyrB and apparently had no affect on the acquisition of FQ resistance. Gibreel et al (1998) amplified the QRDR of *parC* gene and indicated that high levels of ciprofloxacin resistance (up to 125 mg/ml) were due to an Arg-139-Gln mutation in the *parC* product. Conversely, many have not been successful in characterizing the *parC* gene of *C. jejuni* and *C. coli* (Bachoual et al, 2001; Cooper et al, 2002; Payot et al, 2002; Luo et al, 2003; Piddock et al, 2003), and concluded that *Campylobacter* lacks the *parC* gene or quite possibly the gene is not present in all campylobacters.

CmeABC is the most commonly identified efflux pump that contributes to antibiotic resistance in *C. jejuni* and *C. coli* (Lin et al, 2002; Pumbwe and Piddock, 2002; Luo et al, 2003; Ge et al, 2005; Lin et al, 2005). The three proteins CmeA, CmeB, and CmeC are encoded by the three-gene operon (*cmeABC*) (Lin et al, 2005). The role of the *Campylobacter* multidrug efflux gene (*cmeB*) has been examined (Lin et al, 2002; Pumbwe and Piddock, 2002; Lin et al, 2003; Luo et al, 2003; Ge et al, 2005), resulting in marked decreases in FQ resistance after inactivation of the gene. CmeR has been identified as a transcriptional repressor for CmeABC, with mutations in CmeR or the CmeR-binding site impeding repression and resulting in the over-expression of CmeABC and enhanced resistance to multiple antibiotics (Pumbwe et al, 2004; Lin et al, 2005). Pumbwe et al (2005) identified another efflux system, CmeDEF, in the *C. jejuni* NCTC

11168 genome sequence, which was only hypothesized in an earlier study (Pumbwe et al, 2004). However, the overall findings indicated that CmeDEF does not confer resistance to ciprofloxacin.

Efflux pump inhibitors (EPIs) have been utilized to determine the presence of an efflux mechanism resulting in FQ resistance. EPIs were found to be effective in decreasing FQ resistance (Charvalos et al, 1995; Lin et al, 2002; Pumbwe and Piddock, 2002; Payot et al, 2004; Pumbwe et al, 2004), but no significant effect was observed by other researchers (Payot et al, 2002; Corcoran et al, 2005).

1.13.6 Multidrug Resistance

Multidrug resistance (MDR) in *Campylobacter* has been reported in several countries; however, the incidence of a MDR phenotype is a rare event. Typically, research criteria describe MDR as resistance to three (to four) or more antibiotics. The phenotypic MDR profile has been identified in *Campylobacter* isolates of various origin, such as poultry (Oza et al, 2003; Desmonts et al, 2004; Kos et al, 2006; Luangtongkum et al, 2006), swine (van Looveren et al, 2001; Randall et al, 2003; Gebreyes et al, 2005; Schuppers et al, 2005), cattle (Bae et al, 2005; Inglis et al, 2006), as well as in humans (Wang et al, 1984; Tee et al, 1995; Hakanen et al, 2003; Pumbwe et al, 2004). Ge et al (2003) also reported MDR *Campylobacter* in retail meat samples (e.g. turkey, chicken).

Several of these studies have indicated that *Campylobacter* isolates of animal origin tend to have a higher frequency of MDR phenotypes than clinical isolates. *C. coli* isolates tend to acquire resistance to multiple antibiotics more frequently than *C. jejuni* isolates (van Looveren et al, 2001; Bae et al, 2005; Inglis et al, 2006). An active efflux pump system (CmeABC) has been determined the putative cause of the acquisition of

MDR in *Campylobacter* (Charvalos et al, 1995; Lin et al, 2002); however, no correlation was made in a later study (Pumbwe et al, 2005).

1.14 ADENINE AND CYTOSINE METHYLATION AT GATC SITES

DNA modification at specific sites is commonly found in bacterial genomes, and is frequently associated with restriction-modification systems (Low et al, 2001). Methylation of either adenines or cytosines at GATC sites, renders resistance of genomic DNA to enzymes such as *Mbo*I and *Sau*3AI, respectively. *MboI* is sensitive to *dam* methylation and will not cleave GATC sequences that contain methylated adenine, whereas *Sau3*AI is insensitive to *dam* methylation and cleaves all of the DNA samples (Edmonds et al, 1992). This type of DNA modification that has been extensively identified in various bacterial genomes (Barbeyron et al, 1984; Edmonds et al, 1992; Bolstad and Jensen, 1993; Heithoff et al, 2001; Julio et al, 2001), and plays a key role in virulence. DNA methylation provides a mechanism by which additional information is imparted to DNA, occurring at the C-5 or N-4 positions of cytosine and at the N-6 position of adenine (Low et al, 2001).

A study determined that mutations in the DNA adenine methylase (Dam) resulted in mutated strains that were attenuated for virulence (Dueger et al, 2001), but another revealed no significant changes (Honma et al, 2004). DNA methyltransferases mediate the process, which involves the use of S-adenosyl methionine as a methyl donor (Low et al, 2001). DNA methylation has been associated with DNA restriction-modification systems thought to be important in protecting cells from foreign DNA (Low et al, 2001). Restriction-modification systems contain a DNA methylase that protects host DNA

sequences from restriction with cognate restriction enzymes that digest unmodified foreign DNAs (Low et al, 2001).

Chromosomal DNA methylation occurs widely among prokaryotic organisms (Edmonds et al, 1992). Methylated adenines in the GATC sequences were observed in the chromosomal DNAs of various *Campylobacter* species (*C. jejuni, C. coli, C. upsaliensis*) (Edmonds et al, 1992). Cytosine methylation at GATC sites has been described in bacterial pathogens, and found to be characteristic of specific clonal groups (Zheng and Kathariou, 1997; Yildirim et al, 2004). Although information exist on restriction-modification systems involving methylation at GATC sites, there is little information that is available on the presence of either adenine or cytosine modification at GATC sites in *Campylobacter* species.

1.15 GENOTYPING OF *CAMPYLOBACTER*

1.15.1 Introduction

The genus *Campylobacter* comprises a group of closely related Gram-negative bacteria, which primarily colonize the gastrointestinal tracts of a wide variety of host species (Wassenaar and Newell, 2000). Some species reside as commensal organisms, while others exhibit enteropathogenic activity. The diversity within *C. jejuni* and *C. coli* has been well established and is detectable at both the phenotypic and genotypic levels (Wassenaar and Newell, 2000). Genotyping is a highly definitive technique used to characterize *Campylobacter* isolates, as well as other enteropathogens.

Molecular subtyping of isolates within a bacterial species has helped to overcome the many problems faced by microbiologists and epidemiologists. The technique is becoming more useful for investigating sources of infection and routes of transmission in

animals and humans, and has routinely been used to identify outbreak-related strains (Wassenaar and Newell, 2000). Restriction fragment length polymorphism (*fla*-typing), pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) are molecular analyses used for genomic characterization of *Campylobacter* and other foodborne agents.

1.15.2 Restriction Fragment Length Polymorphism (*fla*-typing)

The characteristic corkscrew-like motility of *Campylobacter* is due to the presence of a polar unsheathed flagellum located at one or both ends. The flagellin gene locus of *Campylobacter* species contains two flagellin genes (*flaA* and *flaB*), which are arranged in tandem and are approximately 1.7 kb in size (Wassenaar and Newell, 2000). The genes are highly conserved with 92% identity between *flaA* and *flaB* in individual isolates; however, there is variation between isolates (Newell et al, 2000). Such variation is the reason for formulating a typing scheme that characterizes the flagellin gene of *Campylobacter* isolates.

Advantages of *fla*-typing are a much greater discriminatory power than that of serotyping, its application is simple and quick (involving widely available reagents and equipment), and laboratories worldwide have easily adopted *fla*-typing as a suitable typing method (Newell et al, 2000). *fla*-typing also has very high typeability for *Campylobacter* isolates of various origin, with *C. jejuni* and *C. coli* being equally typeable by the method (Newell et al, 2000). *Campylobacter* strains from humans and various animal hosts have been typed (Alm et al, 1993; Nachamkin et al, 1993; Fitzgerald et al, 2001). Improper DNA extraction technique may affect the ability to type some *Campylobacter* isolates. Disadvantages of *fla*-typing as a typing method are the inability

to compare results among laboratories due to the range of techniques used and genetic instability (Newell et al, 2000). *Campylobacter* spp. are able to undergo recombination between the *fla* genes (Harrington et al, 1997), making that region of the genome highly variable. Also, the number of fragments is sometimes too small for high-resolution subtyping.

1.15.3 Pulsed-Field Gel Electrophoresis (PFGE)

C. jejuni was the first species of the *Campylobacter* genus to be typed by PFGE (Yan et al, 1991); however, the typing method was later used to characterize other *Campylobacter* species (Salama et al, 1992; Fujita et al, 1995; Bourke et al, 1996). Digestion of bacterial chromosomes by restriction enzymes that cleave the DNA infrequently has proved to be a useful typing technique (Wassenaar and Newell, 2000). The application of multiple restriction enzymes can be used to dictate especially stringent criteria for strain identity (Newell et al, 2000). PFGE typing has been a useful tool in determining the genetic relatedness among *Campylobacter* strains from different animal hosts (Fitzgerald et al, 2001; Borck and Pedersen, 2005; Wesley et al, 2005). Chromosomal DNA is embedded in agarose plugs to prevent shearing of the DNA. PFGE separates chromosomal DNA into large fragments (about 20 to 200 kb), and variations in the presence of relevant restriction sites result in genotypic profiles (or macrorestriction profiles) (Wassenaar and Newell, 2000).

The primary advantage of PFGE, compared to other typing methods, is its discriminatory power. The use of multiple enzymes will enhance the discriminatory power of this method. Enzymes that have been used and proved to be efficient for PFGE include *Sma*I, *Sal*I, *Kpn*I, *Apa*I, and *BssH*II. There are several disadvantages imparted

with the use of PFGE as a typing tool. The tedious and time-consuming effort of preparing the agarose plugs containing chromosomal DNA is at the forefront. Some *Campylobacter* strains produce DNase, which will result in DNA degradation if not deactivated. The electrophoresis equipment is specialized and expensive. The enzymes commonly used for typing may not digest the DNA of some *Campylobacter* strains. Although computerized methods have been employed to assist in the interpretation of PFGE results, interpretation can be difficult due to genetic instability leading to discrepancies in the profiles. Electrophoretic conditions vary among laboratories making it difficult to compare results also.

1.15.4 Multilocus Sequence Typing (MLST)

MLST is an unambiguous genotyping method used to characterize bacterial isolates using the sequences of seven housekeeping genes. The technique was proposed by Maiden et al (1998) in which nucleotide sequencing was utilized to assess the alleles of the seven housekeeping genes, with the sequence type of a strain being determined by each allele combination. Internal fragments of each gene are sequenced using an automated DNA sequencer. A comparison is made between the sequences of each fragment to all previously identified sequences (alleles) at that locus; thus, allele numbers are assigned at each of the seven loci (Aanensen and Spratt, 2005). The combination of the seven allele numbers defines the allelic profile of the strain and each different allelic profile is assigned as a sequence type (ST), which is used to describe the strain.

Direct nucleotide sequencing is becoming increasingly automated and consequently is a reasonable alternative method for genotyping bacterial isolates (Wassenaar and Newell, 2000). MLST has been successfully used to characterize various

Campylobacter isolates (Colles et al, 2003; Dingle et al, 2005; Miller et al, 2005; Thakur et al, 2006). MLST is an unambiguous technique that is highly discriminatory, reproducible, and the results can be easily interpreted. Additional advantages of MLST are that there is no need for cultures of bacterial pathogens, the method is easy to standardize, and the results are electronically portable (compare allelic profiles in a global database for each species via the Internet) (Platonov et al, 2000).

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2. Longitudinal study of prevalence and antibiotic resistance profiles of *C. jejuni* and *C. coli* from commercial turkey flocks in eastern North Carolina

2.1 ABSTRACT

Campylobacter is a leading bacterial agent of human diarrheal illness in the United States and other industrialized nations, and contaminated poultry has been identified as a major risk factor for human campylobacteriosis. Currently, limited information exists on colonization of commercial turkeys with *Campylobacter*. In this study, we investigated prevalence and antimicrobial resistance of *Campylobacter* from 15 turkey flocks, representing eight different commercial farms in eastern North Carolina, a major turkey-growing region in the United States. *Campylobacter* was isolated from 1310 of the 1512 turkey fecal samples (87%). *C. jejuni* was recovered from 781 (60%) of the *Campylobacter*-positive samples, and *C. coli* from the remaining 529 (40%). We failed to detect significant seasonal variation in *Campylobacter* prevalence, although the relative prevalence of *C. jejuni* was higher in winter/spring than in summer/fall ($p<0.02$). *C. coli* was more prevalent than *C. jejuni* in young turkeys (brooders, 5-6 weeks), whereas *C. jejuni* predominated in grow-out birds (7-14 weeks) ($p<0.0001$).

The majority of the *Campylobacter* isolates (68% of *C. jejuni* and 54% of *C. coli*) harbored resistance to three or more antibiotics. Although resistance to tetracycline was highly prevalent (85-98%) in both *C. jejuni* and *C. coli*, prevalence of resistance to certain other antibiotics, and prevalence of certain antibiotic resistance profiles, differed markedly between *C. jejuni* and *C. coli*. Resistance to streptomycin was significantly higher in *C. jejuni* than *C. coli* ($p<0.0001$), whereas resistance to the fluoroquinolones (nalidixic acid/ciprofloxacin), or to kanamycin, was more prevalent in *C. coli* ($p<0.0001$).

Resistance to erythromycin was detected frequently in *C. coli*, especially in brooders (35%), but was never detected in *C. jejuni*, from any of the flocks. The findings enhance our understanding of turkey colonization with *Campylobacter*, and can contribute to the assessment of the potential public health threat posed by the presence of the organisms in commercial turkey flocks.

2.2 INTRODUCTION

Campylobacter is the leading bacterial agent of diarrheal illness in the United States and other industrialized countries, with the majority of human cases attributed to *C. jejuni* (85% or more) and *C. coli* (5-10%) (Friedman et al, 2000). *Campylobacter* infections are usually self-limiting, requiring little to no treatment; however, serious sequelae of *Campylobacter* infection include Guillian-Barre syndrome and reactive arthritis (Coker et al, 2002). *C. jejuni* and *C. coli* are often described as "thermophilic" *Campylobacter* species (*C. lari* being the other), having an optimal growth temperature of 41-43˚C. *Campylobacter* is a zoonotic pathogen, being transmitted primarily via contaminated food of animal origin. Meat animals and poultry are frequently colonized with thermophilic campylobacters (Rivoal et al, 2005; Gharst et al, 2006; Jozwiak et al, 2006; Kassa et al, 2006).

Contaminated poultry has been identified as a major risk factor in human campylobacteriosis (Altekruse et al, 2003; Kapperud et al, 2003; Stern et al, 2003; Friedman et al, 2004). Generally, campylobacters colonize poultry without overt signs of infection, and can be readily isolated from the feces of colonized birds (Newell and Fearnley, 2003). Extensive investigations have focused on risk factors for colonization of poultry, especially chickens, with *Campylobacter* (Cory and Atabay, 2001; Allerberger et

al, 2003; Adak et al, 2005; Luber et al, 2006). Current data suggest that the organism is acquired primarily through horizontal transmission, and that the ecology of flock colonization is remarkably complex (Cox et al, 2002; Lee and Newell, 2006).

In comparison to studies focusing on colonization of broilers with *Campylobacter*, similar studies with turkeys are relatively limited. Although some turkey flocks have been shown to be colonized exclusively with *C. jejuni* (Wallace et al, 1998), flocks with a high prevalence of *C. coli* have also been identified (Smith et al, 2004; Lee et al, 2005). At slaughter both *C. jejuni* and *C. coli* can be commonly isolated from the birds (Logue et al, 2003; Wesley et al, 2005; Luangtongkum et al, 2006), and evidence exists that feed withdrawal, catching and transportation to the slaughterhouse are associated with increased prevalence of *C. coli* in certain flocks (Wesley et al, 2005). Surveys of turkey meat at retail have shown prevalence of *C. jejuni* (van Looveren et al, 2001; Zhao et al, 2001; Ge et al, 2003). However, *C. coli* has also been isolated from turkey meat at retail, including strains harboring multiple antibiotic resistance determinants (Ge et al, 2003).

Antibiotics are extensively used in conventional animal production, for therapeutic purposes or to promote growth (van den Bogaard and Stobberingh, 1999; Evans and Wegener, 2003; Phillips et al, 2004), and this has important implications for the emergence and establishment of resistance to antimicrobials in campylobacters that colonize meat animals. Although human diarrheal illness due to *Campylobacter* is typically self-limiting, antibiotic treatment (particularly erythromycin or fluoroquinolones) is necessary for prolonged or more severe cases in special categories of patients. Due to concerns associated with the prevalence of fluoroquinolone resistance

among human isolates, recently several countries have instituted regulations prohibiting the use of fluoroquinolones in poultry production. In the United States, fluoroquinolone use in poultry has not been permitted since July 2005, effective September 2005 (http://www.farmedanimal.net/faw/faw5-34.htm).

The eastern North Carolina region is largely rural, and makes a major contribution to turkey production in the United States. This region also has a high density of swine farms, and frequently the same families grow turkeys as well as swine commercially. This may have important implications in terms of the species of *Campylobacter* that infects turkeys, as well as the antimicrobial resistance of the organisms. Swine are primarily colonized with *C. coli* (Harvey et al, 1999; Saenz et al, 2000; Altekruse et al, 2003; Logue et al, 2003; Thakur et al, 2006), and the antimicrobial resistance profiles of campylobacters from meat animals may differ significantly depending on the type of animal (Aarestrup et al, 1997; Saenz et al, 2000). In earlier studies with turkey flocks in eastern North Carolina, we detected high (80-90%) prevalence of *C. coli* (Smith et al, 2004; Lee et al, 2005), and described the repeated detection of multi-antibiotic resistant *C. coli* strains from successive flocks in one commercial farm (Lee et al, 2005). However, further information on relative prevalence and antibiotic resistance profiles of the two *Campylobacter* species of primary public health concern (*C. jejuni* and *C. coli*) in turkey flocks in this region has been lacking.

To address this gap, we pursued a longitudinal study of prevalence and antibiotic resistance profiles of *C. jejuni* and *C. coli* from 15 commercial turkey flocks, representing eight farms, in eastern North Carolina. These farms were located in a region with

intensive turkey as well as hog production. The turkey farms were in close vicinity to hog farms, and often were operated by common growers.

2.3 MATERIALS AND METHODS

2.3.1 Turkey flocks and sampling times. A total of 15 flocks, representing eight farms, were surveyed during the two year period between October 2003 and October 2005 (Table 2.1). Two different flocks, grown at different times, were surveyed for seven of the farms. Only one flock was surveyed for farm T2, due to the wishes of the grower. All farms were located in eastern North Carolina, within a 100-mile radius of the laboratory. The eight farms were under control of a common integrator (with a common feed mill) but had different water supplies, farm staff, and biosecurity practices. Birds arrived at the farm as day-old poults, and production followed the "all in-all out" system that is standard in the vertically integrated turkey industry, as described before (Smith et al, 2004). Each turkey house typically contained 5,000-6,000 birds. Flocks were taken to market at approximately 14 weeks (hens) or 18-21 weeks (toms).

Antibiotic treatment information was available for T1 (both flocks A and B), T3 (both flocks A and B), T4B, and T7 (both flocks A and B) as follows: T1A, 3-nitro at 4 weeks and tetracycline hydrochloride at 5 weeks; T1B, lincomycin at 10 days, penicillin and 3-nitro at 2.5 weeks, copper sulfate at 7, 9, and 11 weeks; T3A, Baytril® at 1-4 days, penicillin at 2.5 weeks; T3B, penicillin at 3 weeks, lincomycin and amprolium at 4 weeks, copper sulfate at 6 and 9 weeks; T4B, penicillin at 4 days, lincomycin and 3-nitro at 3 weeks, copper sulfate at 4.5, 5.5, and 7 weeks; T7A, penicillin and 3-nitro at 3 weeks, copper sulfate at 8.5 weeks; and T7B, lincomycin and 3-nitro at 5 weeks, copper sulfate at 10 weeks.

2.3.2 Sampling scheme and sample processing. Efforts were made to survey each flock at four different time points: one at 5-6 weeks (brooder stage) and three during grow-out: 7-8 weeks, 10-11 weeks, and 12-14 weeks (Fig. 2.1). Between October 2003 and August 2004, 12 samples were collected at each sampling time, from one turkey house, and the same turkey house was visited each time. For logistic reasons, flocks T1A, T2, and T4B could only be visited at three sampling points (one brooder and two grow-out visits). For flocks surveyed during the remaining of the study period (September 2004 through October 2005) it was possible to obtain 20 samples from each of two houses, totaling 40 samples per visit. For flock T1B, 12 samples were obtained from one brooder house and the grow-out flock could only be surveyed twice (20 samples each from two houses, each time), leading to a total of 92 samples (Table 2.1).

Each house was visually divided into four quadrants and the same number of fresh fecal samples was collected from each quadrant, totaling to either 12 or 20 samples, as described above. Samples were collected with a cotton swab, placed into sterile polypropylene tubes (Corning, New York) and transported on ice to the laboratory. Processing typically was within 24 hours of collection. In the laboratory, direct plating onto modified CCDA (Oxoid, Hampshire, England) was used to isolate *Campylobacter*, and subsequent purifications were done on blood agar medium (Remel, Lenexa, KS), as described (Smith et al, 2004). Cultures were incubated under microaerophilic conditions at 42ºC for 48 hours. At least one culture was purified from a single colony of each culture-positive plate. Long-term storage of the bacteria was in brain heart infusion medium (Becton, Dickson, and Company, Sparks, Maryland) with 20% glycerol, at -80ºC.

2.3.3 Antibiotic susceptibility determinations. Susceptibility to tetracycline, streptomycin, erythromycin, kanamycin, nalidixic acid, and ciprofloxacin was determined as described before (Lee et al, 2005). For susceptibility to tetracycline (10 μg/ml), erythromycin (10 μg/ml), and ciprofloxacin (4 μg/ml), the NCCLS (currently CSLI) protocol was employed, using *C. jejuni* ATCC 33560 (purchased from ATCC) as the quality control strain. Susceptibility to streptomycin, kanamycin, and nalidixic acid was determined as described (Lee et al, 2005) by monitoring growth of the bacteria on Mueller-Hinton Agar plates containing the respective antibiotics (streptomycin, 15 μg/ml; kanamycin, 25 μg/ml; nalidixic acid, 20 μg/ml), following incubation at 42ºC for 48 hours. *C. jejuni* ATCC 33560 was used each time as the quality control strain for each of these antibiotics. No growth of *C. jejuni* ATCC 33560 was observed for any of these antibiotics at the indicated concentrations. Susceptibility of each strain was determined in duplicate, for each of the antibiotics. Results were recorded as resistant (apparent growth) or susceptible (no growth).

2.3.4 DNA extractions and species determinations. Genomic DNA was extracted with the Qiagen DnEasy kit (Qiagen, Valencia, CA), as described by Smith et al (2004). Multiplex PCR was utilized in differentiating *C. jejuni* and *C. coli*, with species-specific primers, *hip* (*C. jejuni*) or *ceuE* (*C. coli*).

2.3.5 Statistical analysis. Statistical analysis employed chi-square tests done through SAS (version 8.02; SAS Institute, Cary, N.C.).

2.4 RESULTS

2.4.1 Frequent recovery of *Campylobacter* from the majority of turkey flocks.

Prevalence data for *Campylobacter* in the 15 flocks are summarized in Table 2.1. With

the exception of one flock from farm T4 (T4A), which yielded only one *Campylobacter*-positive sample, *Campylobacter* was recovered with high frequency (62-100% of the samples) from all other flocks (including T4B, a subsequent flock from farm T4). Of the 1512 turkey fecal samples collected, 1310 (87%) were culture-positive for *Campylobacter* (Fig. 2.2).

Only modest differences in *Campylobacter* prevalence between the different flocks were detected for the seven farms from which two different flocks were surveyed, with the noticeable exception of farm T4, mentioned above. Two other farms (T5 and T6) had noticeably lower prevalence of *Campylobacter* in flock B (81% and 62%, respectively) than in flock A (100% both for T5A and T6A) (Table 2.1). With the exception of farm T4, prevalence of *Campylobacter* tended to decrease from flock A to flock B. This trend was not pronounced, but was consistent among farms, and statistically significant ($p=0.0136$).

2.4.2 Flock-specific prevalence of *C. jejuni* vs. *C. coli*. Of the 1310 *Campylobacter*-positive samples, *C. jejuni* was recovered from 781 (60%) and *C. coli* from 529 (40%) (Fig. 2.3). Other *Campylobacter* species, that would be expected to fail to yield a PCR product with either the *hip* or the *ceu* primers, were not detected. The overall prevalence of *C. jejuni* resulted from high prevalence of this species in samples from certain flocks, specifically T4B (97%) and both flocks of farm T7 (79% and 84%, respectively). Samples from nine of the remaining flocks yielded approximately similar prevalence values for *C. jejuni* and *C. coli* (with the *C. jejuni/C. coli* ratio approximating 1.00), and *C. coli* predominated in three flocks (T1A, T2, T3B). Predominance of *C. coli* appeared

to be specific to the individual flocks, and not the farm, since it was not detected in different flocks from the same farm (T1B, T3A) (Table 2.1).

Excluding farm T4 (which had unusually low prevalence of *Campylobacter* in flock A), two farms exhibited major changes in the relative proportion of *C. jejuni* and *C. coli* between different flocks. In farm T1, the dominant species changed from *C. coli* to *C. jejuni*, and in farm T7, *C. jejuni* outnumbered *C. coli* considerably more in the second flock ($p<0.0001$) (Table 2.1).

2.4.3 Relative prevalence of *C. jejuni* and *C. coli* varies significantly between brooders and grow-out birds. The prevalence of *C. jejuni* was lower (46%) in the brooders (5 to 6 weeks of age), than in the grow-out phase (56 to 69%), when the birds were 7 to 14 weeks of age. An inverse trend was noted with *C. coli,* which was more prevalent in brooders (54%) than in grow-out birds (31 to 38%) (Table 2.2 and Fig. 2.4). The differences in the relative prevalence of *C. jejuni* and *C. coli* between brooders (5-6 weeks) and grow-out birds (7-14 weeks) were statistically significant ($p<0.0001$). With both species, relative prevalence as well as total prevalence (Fig. 2.5) remained constant throughout the grow-out phase, suggesting that the change in relative and total prevalence took place upon the transition from the brooder to the grow-out phase.

2.4.4 Lack of significant seasonal impact on total prevalence of *Campylobacter* in turkey fecal samples. The survey periods were divided into two seasonal periods, corresponding to major differences in temperature in this region: winter/spring (December-May) and summer/fall (June-November) months. We failed to detect significant differences in total prevalence of *Campylobacter* in turkey fecal samples collected in winter/spring (87%) versus those collected in summer/fall (86%) (Table 2.3

and Fig. 2.6). However, the relative prevalence of *C. jejuni* was significantly higher in winter/spring than in summer/fall (*C. jejuni/C. coli* ratio 1.81 and 1.29, respectively) ($p=0.0284$). Thus, even though total *Campylobacter* prevalence was similar in winter/spring and summer/fall samples, and *C. jejuni* was overall more prevalent than *C. coli* during both seasonal periods, the likelihood of a sample yielding *C. jejuni* was higher in winter samples, and conversely with *C. coli*.

2.4.5 Antibiotic susceptibility varies between *C. jejuni* and *C. coli*, and in isolates from birds of different age groups. Of the antibiotics that were tested (tetracycline, streptomycin, erythromycin, kanamycin, nalidixic acid and ciprofloxacin), tetracycline was the only one for which prevalence of resistance was similar in *C. jejuni* and *C. coli*. Resistance to this antibiotic was high in *C. jejuni* and *C. coli* from birds of all four age groups, ranging from 86 to 98% (Table 2.4 and Fig. 2.7).

Prevalence of resistance to the other tested antibiotics tended to be higher in *C. coli* than *C. jejuni*, with the notable exception of resistance to streptomycin, which was significantly more prevalent in *C. jejuni* (ranging from 84 to 96%) than in *C. coli* (24 to 39%) ($p<0.0001$) (Table 2.4 and Fig. 2.7). Overall prevalence values of resistance to kanamycin or to fluoroquinolones (nalidixic acid/ciprofloxacin) were significantly higher in *C. coli* than in *C. jejuni* ($p<0.0001$). Resistance to erythromycin was encountered exclusively in *C. coli* (18-35%), with none of the 781 *C. jejuni* isolates from the turkey fecal samples harboring resistance to this antibiotic (Table 2.4 and Fig. 2.7). A similar trend was noted in regards to the overall total prevalence of antibiotic resistance among *C. jejuni* and *C. coli* isolates (Fig. 2.8).

Prevalence of antibiotic resistance in *C. coli* decreased significantly with increasing age of the flock for all tested antibiotics except tetracycline, which remained constant (Table 2.4 and Fig. 2.7). The decline in prevalence of resistance to streptomycin and erythromycin was gradual, but *C. coli* isolated from samples of 12-14 week-old birds were much less likely to be resistant to these two antibiotics than isolates from 5-6 week brooders ($p=0.0075$ and $p=0.0020$ for streptomycin and to erythromycin, respectively). Marked decreases in prevalence of resistance to kanamycin, nalidixic acid and ciprofloxacin were observed as birds aged, with prevalence values being noticeably higher in *C. coli* from birds of 5-6 weeks (kanamycin) or 5-8 weeks (nalidixic acid/ciprofloxacin) than in those from older birds (Table 2.4 and Fig. 2.7).

In contrast to the decreasing prevalence of antibiotic resistance observed in the *C. coli* isolates as the flocks aged, such a trend was not consistently detected with *C. jejuni*. Tetracycline was the only antibiotic to which prevalence of resistance, albeit overall high (86-98%), nonetheless declined with increasing age of the flock ($p=0.0016$). The most noticeable change in prevalence of resistance as birds aged concerned resistance to nalidixic acid and ciprofloxacin, the prevalence of which was significantly lower in brooders (5-6 weeks) than in grow-out birds ($p<0.01$) (Table 2.4 and Fig. 2.7).

2.4.6 Major antibiotic resistance profiles differ markedly between *C. jejuni* and *C. coli*. Numerous antibiotic resistance profiles were observed among *C. jejuni* and *C. coli* isolates from turkeys, including 14 profiles in *C. jejuni* and 18 in *C. coli* (Table 2.5). However, only four profiles (encountered in >10% of the isolates) were identified in each species. In *C. jejuni*, the four major profiles included TSKQ (tetracycline, streptomycin, kanamycin, nalidixic acid and ciprofloxacin; 34%), TSQ (tetracycline, streptomycin,

nalidixic acid and ciprofloxacin; 21%), TSK (tetracycline, streptomycin, kanamycin; 11%), and TS (tetracycline, streptomycin; 19%). The predominant *C. coli* profiles included the multi-drug resistance (TSEKQ) profile (resistance to tetracycline, streptomycin, erythromycin, kanamycin, nalidixic acid and ciprofloxacin; 22%), TKQ (tetracycline, kanamycin, nalidixic acid and ciprofloxacin; 20%), TQ (tetracycline, nalidixic acid and ciprofloxacin; 21%), and TK (tetracycline, kanamycin; 11%). It is notable that none of the major profiles were the same in *C. jejuni* and *C. coli*. Since none of the *C. jejuni* isolates harbored resistance to erythromycin, all resistance profiles that included resistance to this antibiotic were unique to *C. coli* (Table 2.5).

Isolates harboring resistance to just one of the tested antibiotics (with nalidixic acid and ciprofloxacin being considered one antibiotic class) were rare among either *C. jejuni* or *C. coli*, and represented no more than 7% of the isolates. Only four *C. jejuni* and one *C. coli* isolates (0.5% and 0.2% of each population) were susceptible to the entire panel of antibiotics, respectively (Table 2.5). In contrast, multi-drug resistant isolates, defined as those harboring resistance to three or more of the antibiotics (with nalidixic acid and ciprofloxacin being considered one antibiotic class) were frequent, representing 69% and 54% of *C. jejuni* and *C. coli*, respectively.

2.5 DISCUSSION

Relatively few reports have described longitudinal studies of colonization of turkeys with *Campylobacter* at different time points during the pre-harvest phase (Wallace et al, 1998; Smith et al, 2004; Lee et al, 2005). In earlier studies of selected flocks in eastern North Carolina, a major turkey-growing region in the United States, we

showed that *Campylobacter* colonization was extensive by 2-3 weeks of age of the birds, and that most isolates were *C. coli*. Furthermore, examination of one farm over successive flocks showed repeated presence of closely related strains that were resistant to multiple antibiotics (Smith et al, 2004; Lee et al, 2005).

The current study of 15 flocks confirmed the extensive prevalence of *Campylobacter* in commercial turkeys in this region. However, *C. coli* was only found to be predominant in three of the flocks, and *C. jejuni* was overall the dominant species (60% of the samples). The reasons for the apparent decrease in relative prevalence of *C. coli* (and increase in *C. jejuni*), in comparison to the earlier studies are unclear at this time. In eastern North Carolina, turkey production is in a "swine-dense" region, and the turkey farms that participated in this study were in close proximity to swine farms, often under the care of a common grower. Hence, the potential exists for transfer of the organism from swine, which are commonly colonized with *C. coli*, to turkey operations. The current data, however, suggest that the turkey flocks, in spite of the close proximity of swine farms, were frequently colonized with *C. jejuni*. Additional surveillance of the relative prevalence of these two species in commercial turkeys will be needed in order to identify possible temporal shifts in prevalence. On the basis of our communications with representatives of the turkey integrator, and with the growers themselves, no clear changes in production could be identified between the current flocks and those investigated in earlier studies (Smith et al, 2004; Lee et al, 2005).

Relative prevalence of *C. jejuni* versus *C. coli* has been noticeably variable in different studies. For instance, birds were found to be colonized by *C. jejuni* (exclusively) in a study of five flocks in the United Kingdom (Wallace et al; 1998). In

contrast, in a study of five flocks from Iowa, *C. coli* was frequently identified in samples from birds shortly prior to slaughter, and its relative prevalence in three flocks was found to increase following feed withdrawal and transport to the slaughterhouse (Wesley et al, 2005). Samples from birds after slaughter revealed high prevalence of *C. jejuni* in one plant, but *C. coli* in another (Logue et al, 2003); intestinal (cecal) samples from four flocks of conventionally grown turkeys yielded primarily *C. coli*, whereas *C. jejuni* was predominant in intestinal samples from organically grown turkeys (five flocks) (Luangtongkum et al, 2006). Such variable findings suggest that turkey flocks may differ markedly in their relative colonization by *C. jejuni* and *C. coli*, possibly depending on region, production practices, and currently unidentified flock-specific factors, and suggest caution in making generalized conclusions about the relative prevalence of either of these species in this avian host. Furthermore, caution needs to be applied in interpreting findings from different types of samples. Data from our laboratory suggest that relative prevalence of *C. jejuni* versus *C. coli* may differ in samples from the cecum versus fecal samples (S. Kathariou, R. Siletzky and D. K. Carver, unpublished findings) and findings from other studies suggest differences between cloacal samples and samples from the cecum, with *C. coli* being more prevalent in the cecum (Wesley et al, 2005). Fecal sampling was employed in this study not only because of its obvious logistical advantages but also because, in our opinion, it allowed a representative sampling of organisms present in the entire gastrointestinal tract, and might avoid site-specific bias obtained by samples focusing on the cecum.

Surveys of different (typically non-consecutive) flocks grown at the same farm revealed high prevalence of *Campylobacter*, identified on only two farms, T4 and T6,

that had marked differences in prevalence from one flock to the other. The reasons for the extremely low prevalence of colonization of birds from the first surveyed flock from farm T4 are unknown. Studies with broilers have indicated the occasional presence of *Campylobacter*-free flocks (Humphrey et al, 1993; Stern et al, 2001; Rasschaert et al, 2006). Interestingly, when farm T4 was surveyed again, the flock (T4B) was extensively colonized, and the majority (86%) of the isolates were *C. jejuni*. Similarly high prevalence of *C. jejuni* was also noted in both flocks of another farm (T7). The reasons for some flocks deviating noticeably from the average in prevalence of *C. jejuni* versus *C. coli* remain unknown. Among other factors, treatment regimens of the flock may impact relative species prevalence, since resistance to certain antibiotics was found to be significantly more common in one species versus the other. For instance, treatment of the flock with erythromycin would be expected to result in the prevalence of *C. coli*, whereas streptomycin treatment might be expected to promote the prevalence of *C. jejuni*.

Overall, *C. coli* was more prevalent in young turkeys (brooders) than in grow-out birds (7-14 weeks), whereas the prevalence of *C. jejuni* appeared to increase as the birds aged. Such findings are intriguing, and suggest age-related associations in prevalence of the two species. It remains unknown whether these may be due to intrinsic host factors, such as the maturing immune system and changing gastrointestinal microbiota of the birds, or to other factors that also change in commercial turkey production, such as different feed and antimicrobial exposure regiments (either in growth promoters or therapeutically) in brooders versus grow-out birds.

In contrast to results with broilers (Wedderkopp et al, 2001; Patrick et al, 2004) overall prevalence of *Campylobacter* in the turkey flocks was not significantly influenced

by season. The apparent difference between turkeys and broilers regarding seasonal impact should be investigated further over additional flocks and years, with inclusion of flocks from different regions, to determine whether the lack of seasonal impact is an intrinsic attribute of turkey colonization. If this proves to be the case, one may speculate that at least some of the key reservoir(s) of *Campylobacter* for turkeys and broilers may differ, with seasonal influence strongly impacting broiler-specific reservoirs. Several studies have implicated vectors such as flies, whose abundance and movement are clearly season-dependent, in colonization of broiler flocks (Shane et al, 1985; Hald et al, 2004; Nichols, 2005) and similar evidence was recently described in cattle (Adhikari et al, 2004). Similar studies with turkeys are currently lacking. It is noteworthy, however, that even though overall prevalence of *Campylobacter* was not season-dependent, the *C. jejuni*/*C. coli* ratio was higher in the colder months. Turkeys are prone to heat stress and diarrheal (mostly viral) illness during the summer in eastern North Carolina, and it is possible that such conditions make the birds more susceptible to colonization by *C. coli*. In earlier studies we also found that *C. coli* was especially predominant in young (1-4 weeks old) turkeys that were experiencing severe episodes of diarrheal illness during the summer months in this region (Lee et al, 2005). Further studies are needed to identify and characterize possible host susceptibility factors that may impact colonization of turkeys with *C. jejuni* versus *C. coli*.

In this study, remarkably high prevalence of resistance to tetracycline was observed among both *C. jejuni* and *C. coli*, from all *Campylobacter*-positive turkey flocks. Tetracycline is used extensively in poultry production systems as a feed additive and for therapeutic purposes (Chopra and Roberts, 2001), and the high prevalence may

reflect such use. On the other hand, the tetracycline resistance determinant *tetO* can be disseminated conjugatively among campylobacters, even in the absence of selection for resistance to the antibiotic (Avrain et al, 2004). The frequent detection of tetracycline resistance among *Campylobacter* isolated from organically raised birds (Cui et al, 2005; Luangtongkum et al, 2006) also suggest that resistance to this antibiotic is stably maintained in *Campylobacter*, even in the absence of antibiotic use.

We observed several intriguing differences in prevalence of resistance to certain antibiotics in *C. jejuni* versus *C. coli*. Streptomycin resistance was significantly higher in *C. jejuni,* whereas resistance to kanamycin or to nalidixic acid/ciprofloxacin was more prevalent in *C. coli*. Erythromycin resistance was encountered among 27% of the *C. coli* isolates, but was not detected in any of the 781 tested *C. jejuni* isolates. The species-dependent prevalence of erythromycin resistance has also been reported in several studies, which in agreement with our results, indicate that *C. coli* is significantly more likely to harbor resistance to this antimicrobial than *C. jejuni* (Moore et al, 1996; Saenz et al, 2000; Payot et al, 2004). The higher prevalence of nalidixic acid/ciprofloxacin resistance among *C. coli* than *C. jejuni* is also in agreement with other studies (Thwaites and Frost, 1999; Savasan et al, 2004; Kurincic et al, 2005). On the other hand, the significantly higher prevalence of resistance to streptomycin among *C. jejuni* (than *C. coli*) was unexpected. In other studies, *C. coli* (primarily from swine) had high rates of resistance to streptomycin (Aarestrup et al, 1997; Regula et al, 2003). In our own studies, prevalence of streptomycin resistance among *C. coli* from swine in eastern North Carolina (58%) was indeed higher than that of *C. coli* from turkeys (31%) (S. Wright, R. Siletzky and S. Kathariou, unpublished findings), albeit not as high as the prevalence rate

(91%) in *C. jejuni* from turkeys in the current study. The reasons for the differential prevalence to these antibiotics (especially erythromycin and streptomycin) in *C. jejuni* versus *C. coli* from turkeys remain unidentified.

Intriguing trends in antibiotic resistance were observed in both *C. jejuni* and *C. coli* in association with age of the birds. In *C. jejuni*, resistance to tetracycline, although remaining high throughout the lifetime of the flock, showed a decreasing trend (from 98% in brooders to 86% in 12-14 week old birds). In contrast, in *C. coli* tetracycline resistance remained fairly constant, but decreasing trends were observed with all three other antibiotic classes, even though resistance rates remained substantial for all (for example, erythromycin and nalidixic acid/ciprofloxacin resistance were 35% and 84%, respectively, in brooders, but 18% and 68%, respectively, in 12-14 week old birds). The only noticeable increase in prevalence of antibiotic resistance as birds aged involved fluoroquinolone resistance in *C. jejuni* (from 31% in 5-6 week old brooders to 71% in 12-14 week old birds, close to marketing).

Decreases in prevalence of resistance to certain antibiotics may reflect antimicrobial use at the farm, which tends to be lesser as the birds age. Alternatively, the trends may reflect different fitness of strains with different antimicrobial resistance profiles. This may contribute especially to the observed increase in prevalence of nalidixic acid/ciprofloxacin resistance in *C. jejuni* as the birds aged. Experimental evidence suggests that certain fluoroquinolone-resistant *C. jejuni* strains had higher fitness than their fluoroquinolone-susceptible counterparts in regard to their infection and persistence in chickens (Luo et al, 2005). In this context, it is intriguing that in *C. coli* from turkeys prevalence of fluoroquinolone resistance decreased with bird age. Such

differences may suggest that resistance to these antibiotics may differentially affect fitness in *C. jejuni* and *C. coli* colonizing turkeys.

Multi-drug resistant isolates were commonly detected among both *C. jejuni* and *C. coli*; conversely, strains with resistance to none, or only one of the tested antibiotics, were rare in both species. Our personal communications with the company representatives indicated that clearly not all of the antibiotics to which the organisms were resistant were in current use. Higher colonization potential or environmental fitness in the conventional turkey production ecosystem may at least partially account for the observed prevalence of multi-drug resistant strains, as has been speculated by others (Luangtongkum et al, 2006). Clearly further studies need to be done to elucidate the mechanisms mediating the prevalence of *C. jejuni* and *C. coli* strains with different antibiotic resistance profiles in the turkey flocks.

In conclusion, our findings suggest that commercial turkey flocks in eastern North Carolina are commonly colonized with both *C. jejuni* and *C. coli*, and that the relative prevalence of these two species is flock-specific, and varies as the birds aged from brooder to grow-out. In addition, prevalence of resistance to certain antimicrobials varies markedly according to species (*C. jejuni* versus *C. coli*), and according to age of the birds. Such findings describe, to our knowledge for the first time, complex longitudinal patterns in colonization and antibiotic resistance of *C. jejuni* and *C. coli* that colonize commercial turkeys, and point to the need for further studies to better understand the risk factors involved. Such understanding would be required for better evaluation of the public health burden associated with presence and antibiotic resistance of these organisms in the commercial turkey production system.

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Table 2.1 Prevalence of *Campylobacter*, *C. jejuni*, and *C. coli* in turkeys produced in eastern North Carolina.

Farm[1]	Survey Period	*Campylobacter*	*C. jejuni*	*C. coli*
T1A	Oct. 2003-Dec. 2003	36/36 (100)	13/36 (36)	23/36 (64)
T1B	June 2004-Aug. 2004	84/92 (91)	48/92 (52)	36/92 (39)
T2	Nov. 2003-Dec. 2003	35/36 (97)	11/36 (30)	24/36 (67)
T3A	Feb. 2004-March 2004	47/48 (98)	22/48 (46)	25/48 (52)
T3B	June 2005-Aug. 2005	143/160 (89)	55/160 (34)	88/160 (55)
T4A	March 2004-April 2004	1/48 (2)	1/48 (2)	0/48 (ND)
T4B	July 2004-Aug. 2004	35/36 (97)	31/36 (86)	4/36 (11)
T5A	March 2004-May 2004	48/48 (100)	26/48 (54)	22/48 (46)
T5B	Aug. 2005-Oct. 2005	130/160 (81)	75/160 (47)	55/160 (34)
T6A	March 2004-May 2004	48/48 (100)	26/48 (54)	22/48 (46)
T6B	Aug. 2005-Oct. 2005	99/160 (62)	51/160 (32)	48/160 (30)
T7A	Sep. 2004-Nov. 2004	157/160 (98)	127/160 (79)	30/160 (19)
T7B	Jan. 2005-March 2005	138/160 (86)	134/160 (84)	4/160 (2)
T8A	Oct. 2004-Nov. 2004	150/160 (94)	75/160 (47)	75/160 (47)
T8B	Dec. 2004-Feb. 2005	160/160 (100)	86/160 (54)	74/160 (46)

1 Letters (A,B) indicate repeated surveys at the same farm, during the indicated time periods

Table 2.2 Prevalence of *C. jejuni* and *C. coli* isolates for the four sampling time points

Sampling Time Points	*Campylobacter*-positive samples	*C. jejuni*	*C. coli*
1 (n=376)[1]	282/376 (75)[2]	129/282 (46)	153/282 (54)
2 (n=392)	346/392 (88)	210/346 (61)	136/346 (39)
3 (n=376)	326/376 (87)	204/326 (63)	122/326 (37)
4 (n=368)	356/368 (97)	238/356 (67)	118/356 (33)

1 n indicates the total number of fecal samples collected per time point
2 Percentages in parentheses

Table 2.3 Seasonal impact on recovery of *C. jejuni* and *C. coli* from turkey fecal samples

	Winter/Spring (n=548) [1]	**Summer/Fall (n=965)** [1]	**Total**
C. jejuni	306 (56)[2]	475 (49)	781
C. coli	171 (31)	358 (37)	529
Total[3]	477 (87)	833 (86)	1310

1 n indicates total number of samples collected per seasonal period
2 Percentages are indicated in parentheses
3 Total number of *Campylobacter*-positive samples per seasonal period

Table 2.4 Antibiotic resistance among turkey-derived *C. jejuni* and *C. coli* isolates at different sampling ages of the flocks

		Resistance to				
Source	**Sampling Time[1]**	**Tet**	**Str**	**Em**	**Kan**	**Nal/Cipro**
C. coli	1 (n=153)	143 (93)[2]	60 (39)	54 (35)	115 (75)	128 (84)
	2 (n=136)	122 (90)	39 (29)	36 (26)	87 (64)	121 (89)
	3 (n=122)	117 (96)	39 (32)	32 (24)	71 (58)	86 (70)
	4 (n=118)	110 (92)	27 (24)	22 (18)	60 (52)	80 (68)
	Total=529	492 (93)	165 (31)	144 (27)	333 (63)	415 (78)
C. jejuni	1 (n=129)	126 (98)[2]	124 (96)	0 (0)	55 (43)	40 (31)
	2 (n=210)	196 (94)	174 (84)	0 (0)	113 (54)	134 (64)
	3 (n=204)	181 (89)	189 (93)	0 (0)	116 (57)	141 (69)
	4 (n=238)	203 (86)	223 (95)	0 (0)	101 (42)	168 (71)
	Total=781	706 (91)	710 (91)	0 (0)	385 (49)	483 (62)

1 Samples at sampling times 1,2,3, and 4 were obtained when turkeys were 5-6,7-8,10-11, and 12-14 weeks of age, respectively; n indicates total number of *C. jejuni* or *C. coli* isolates from the indicated sampling times

2 Percentages are indicated in parentheses

Table 2.5 Antibiotic resistance profiles in turkey-derived *C. jejuni* and *C. coli* isolates

AB^R Profile	*C. jejuni*		*C. coli*	
No resistance	4	(0.5%)[1]	1	(0.2%)[1]
E	0	(0%)	0	(0%)
K	0	(0%)	1	(0.2%)
Q	16	(2%)	30	(6%)
S	47	(6%)	0	(0%)
T	3	(0.4%)	35	(7%)
EK	0	(0%)	0	(0%)
KQ	2	(0.3%)	3	(0.6%)
SE	0	(0%)	0	(0%)
SQ	3	(0.4%)	2	(0.4%)
TE	0	(0%)	2	(0.4%)
TK	11	(1%)	58	(11%)
TQ	6	(0.8%)	109	(21%)
TS	148	(19%)	0	(0%)
SKQ	1	(0.1%)	0	(0%)
TEK	0	(0%)	1	(0.2%)
TEQ	0	(0%)	1	(0.2%)
TKQ	27	(3%)	107	(20%)
TSE	0	(0%)	0	(0%)
TSK	83	(11%)	7	(1%)
TSQ	167	(21%)	16	(3%)
TEKQ	0	(0%)	16	(3%)
TSEK	0	(0%)	9	(2%)
TSKQ	261	(34%)	16	(3%)
TSEKQ	0	(0%)	115	(22%)

1 Percentages in parentheses

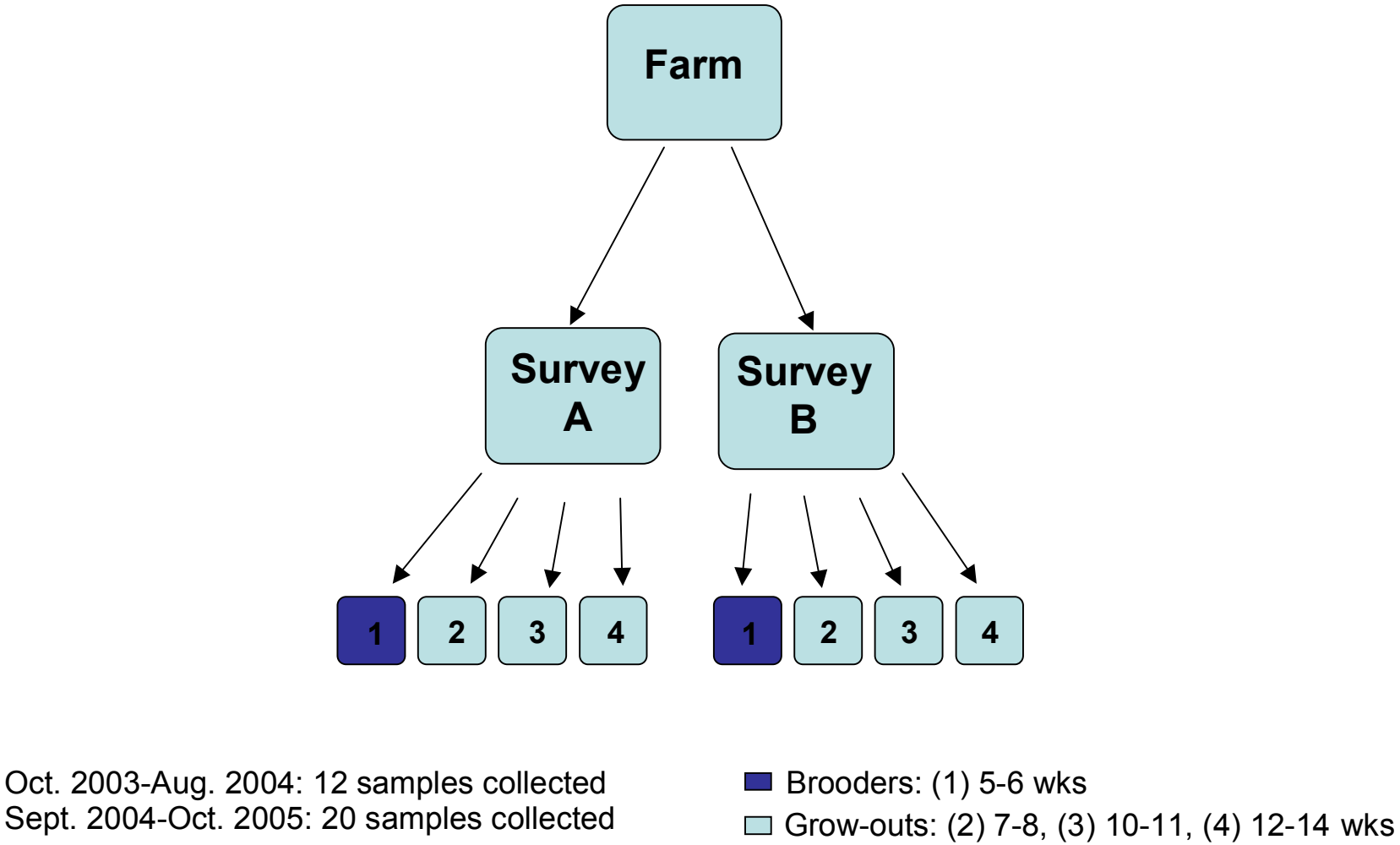

Figure 2.1 Flow diagram of sampling scheme on surveyed farms

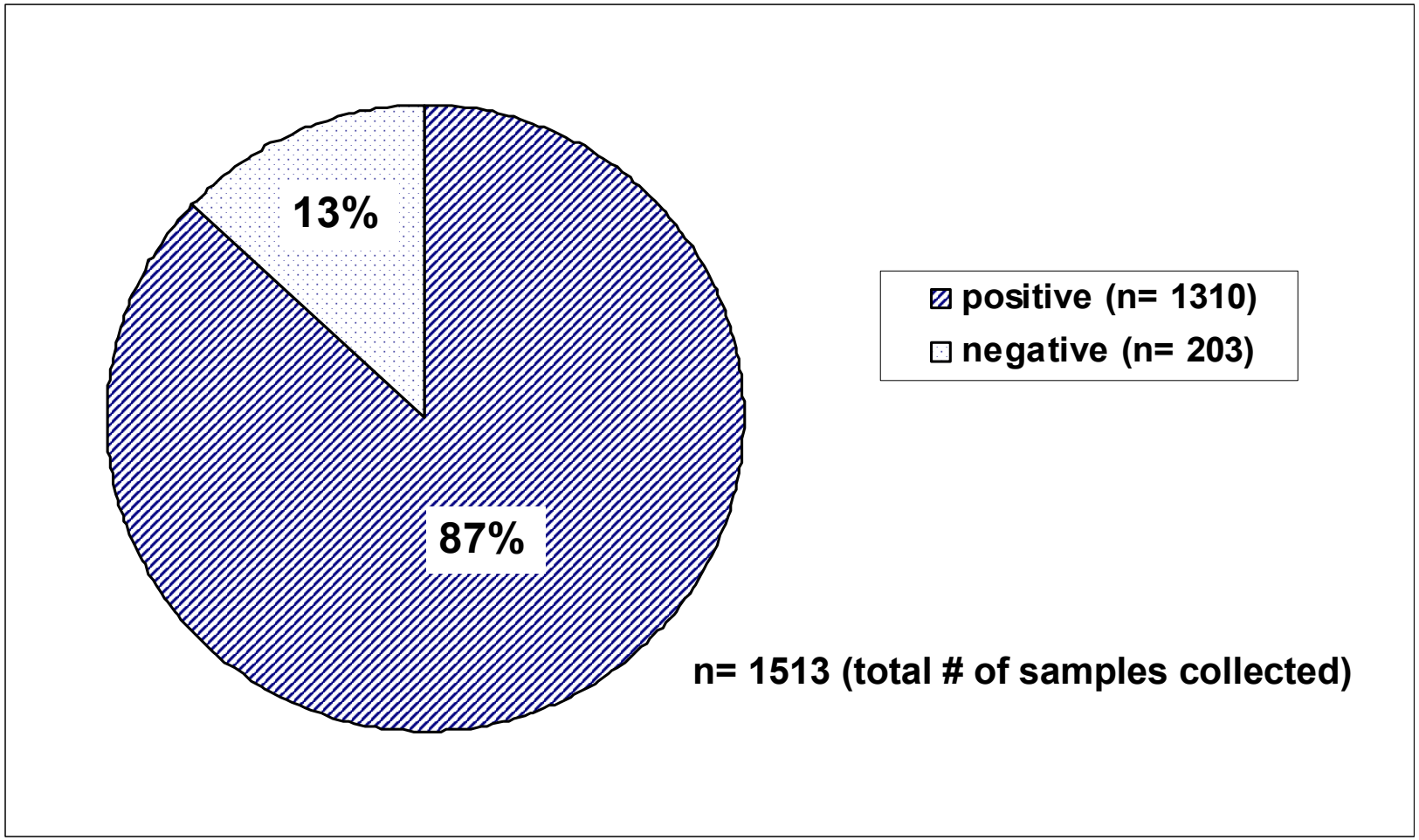

Figure 2.2 Recovery of *Campylobacter* from turkey fecal samples

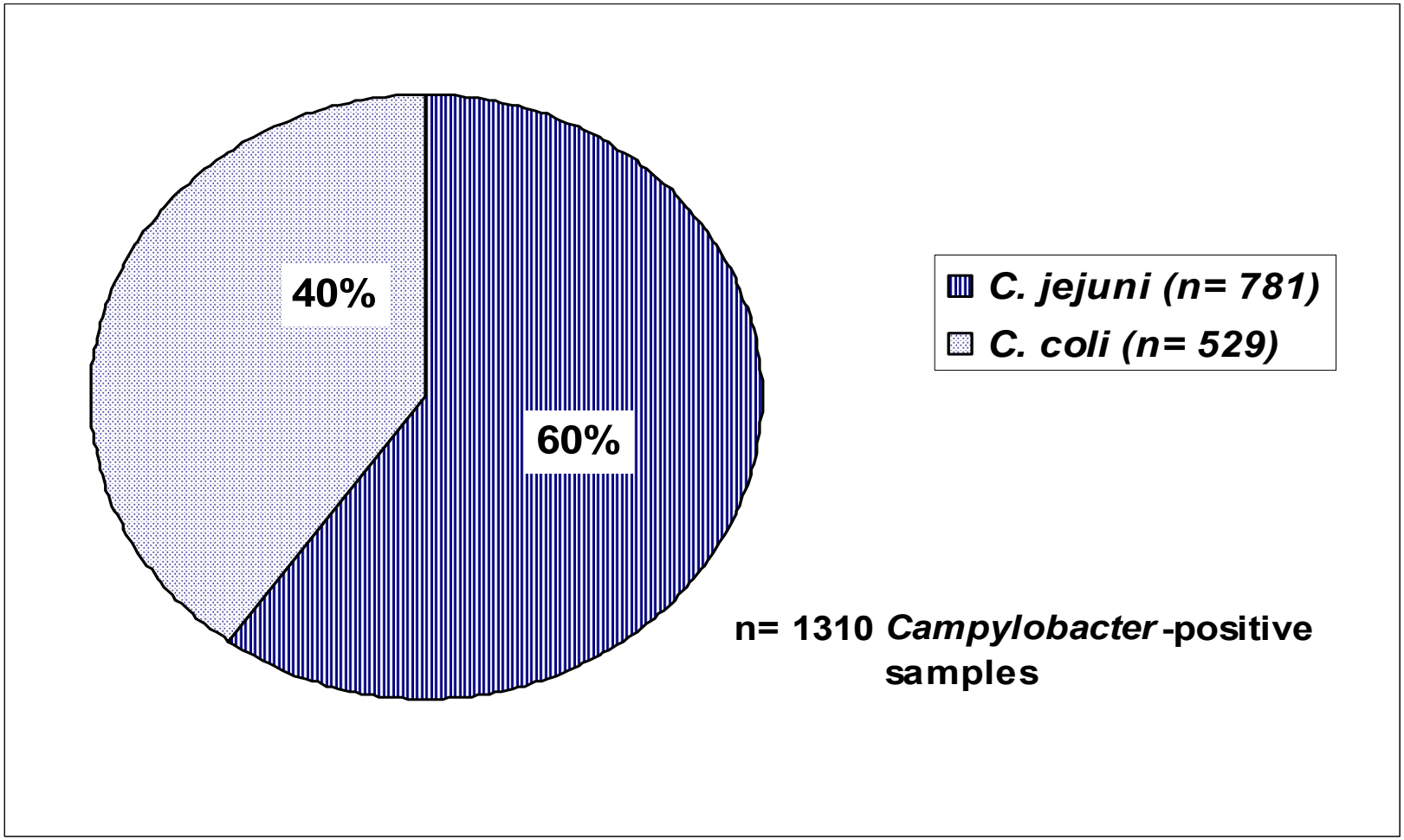

Figure 2.3 Prevalence of *C. jejuni* vs. *C. coli* among flocks

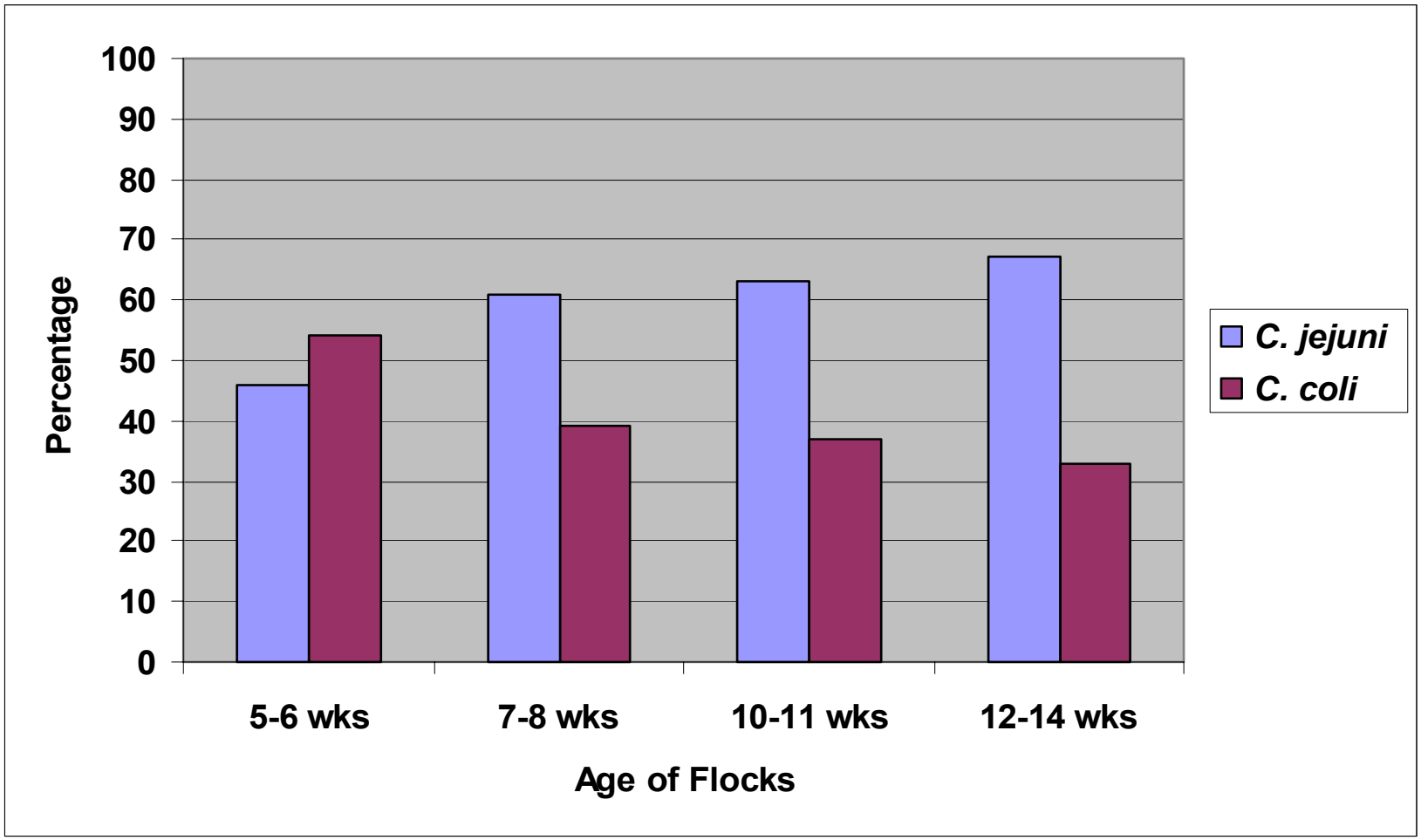

Figure 2.4 Relative prevalence of *C. jejuni* vs. *C. coli* in turkeys at different ages

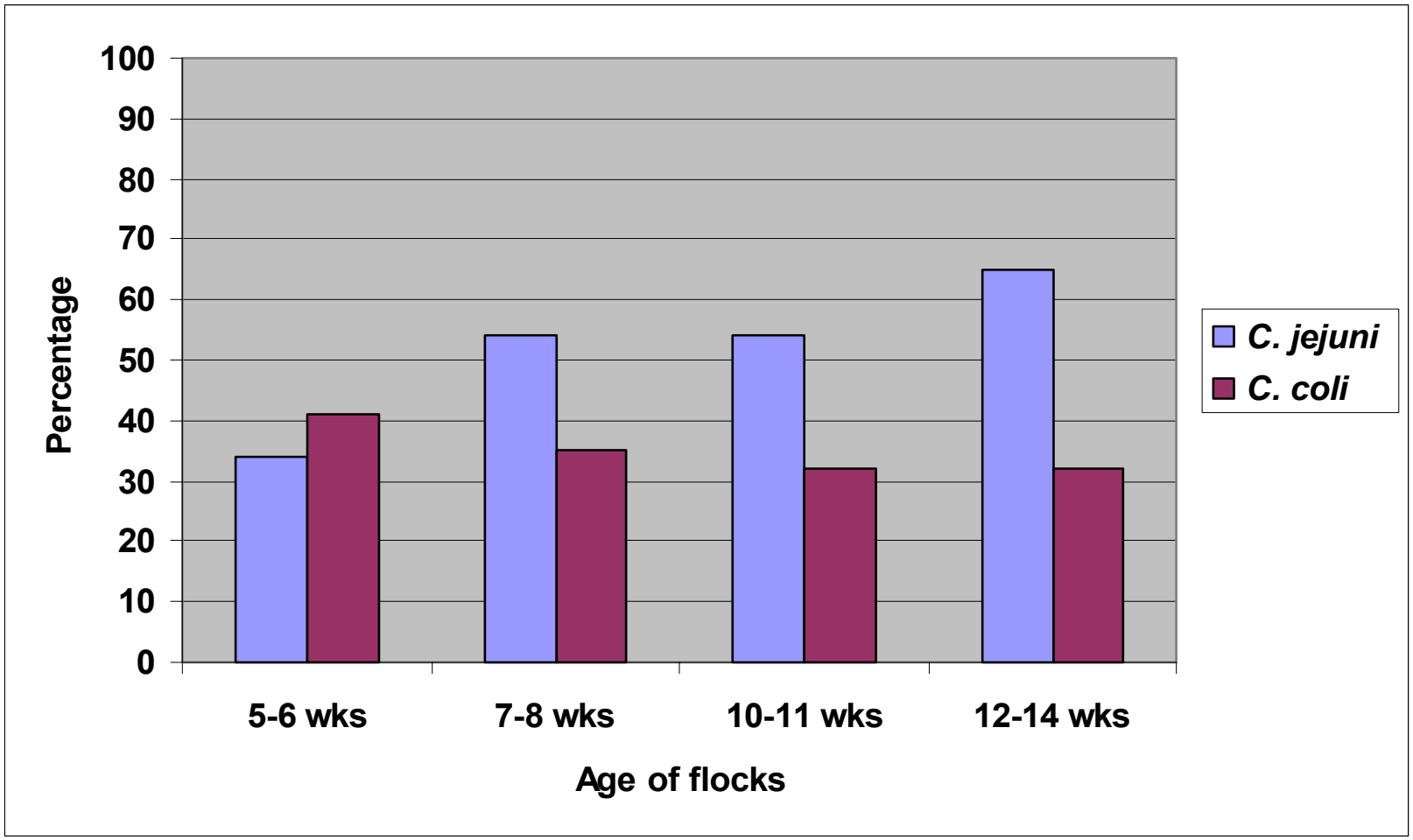

Figure 2.5 Total prevalence of *C. jejuni* vs. *C. coli* in turkeys at different ages

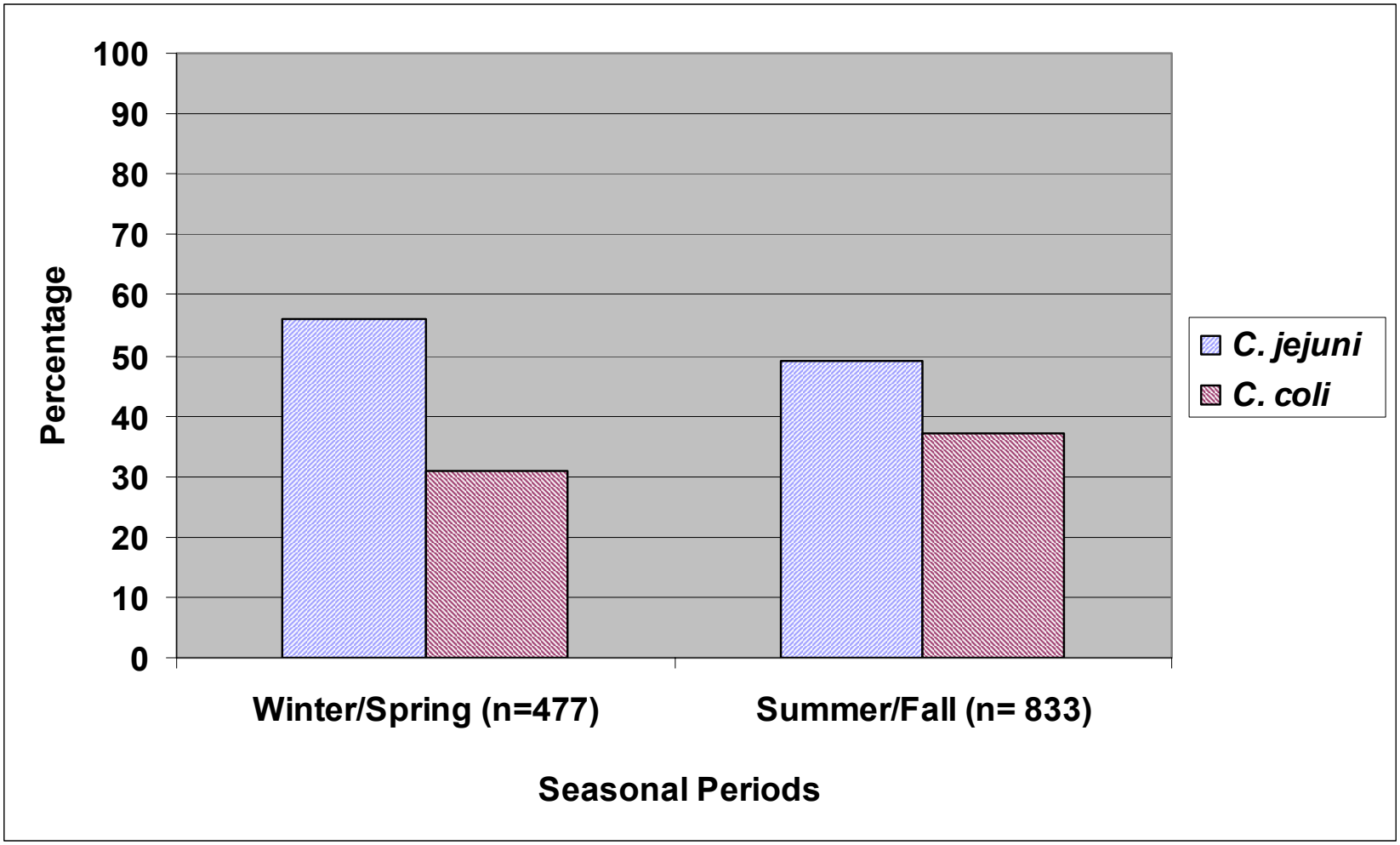

Figure 2.6 Seasonal impact on recovery of *C. jejuni* and *C. coli* from turkeys

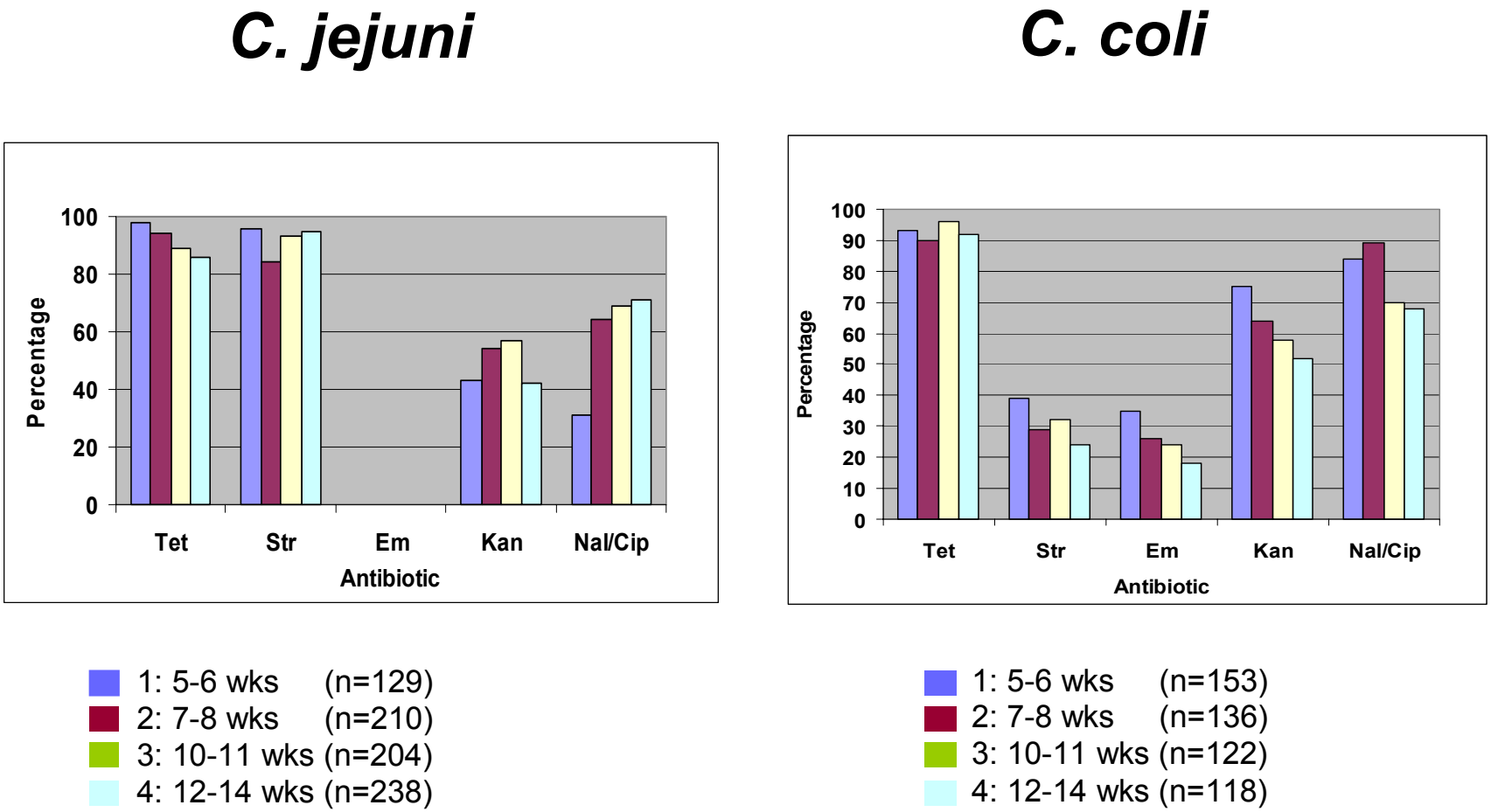

Figure 2.7 Antibiotic resistance among turkey-derived *C. jejuni* and *C. coli* isolates at different ages

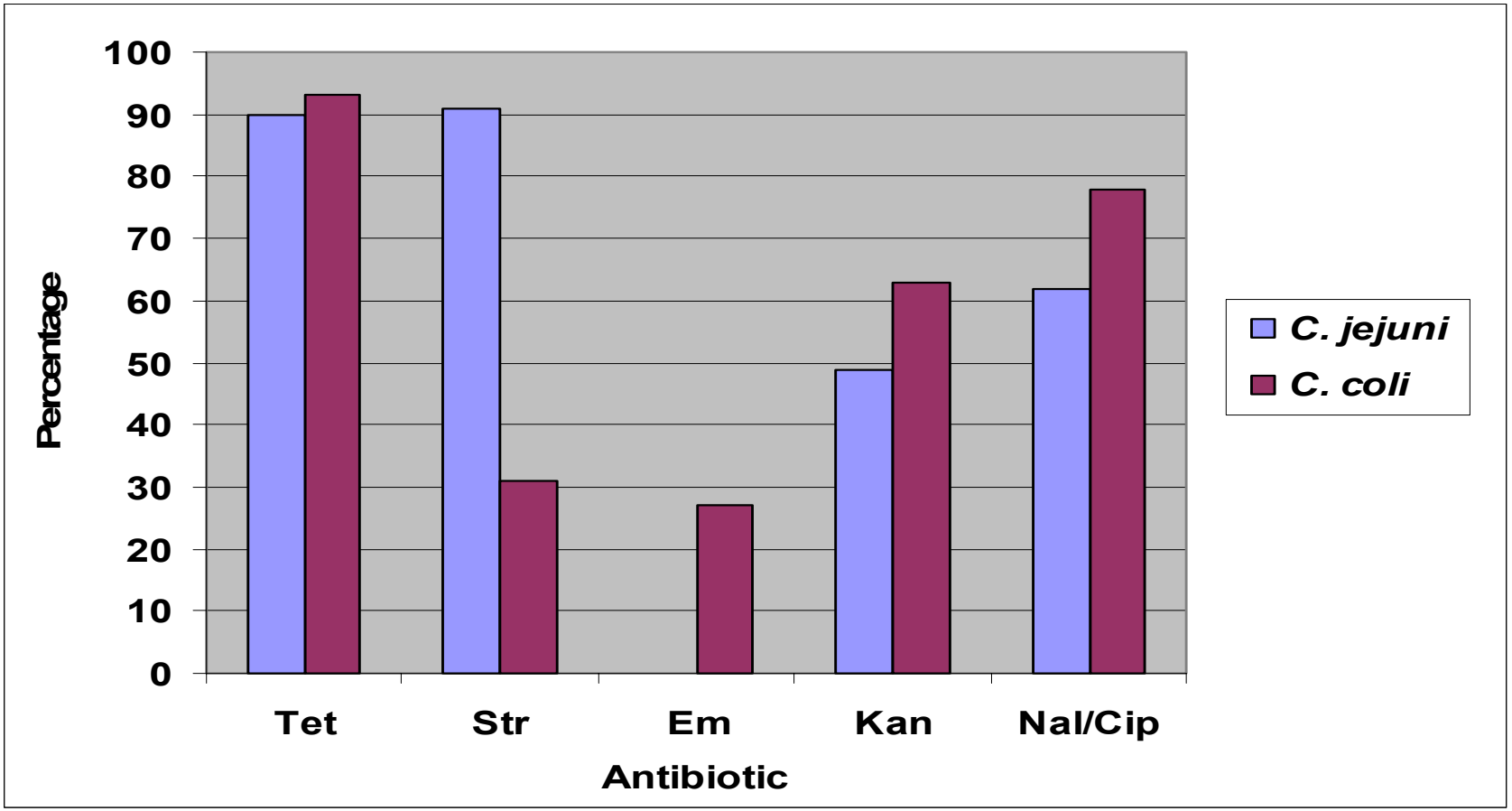

Figure 2.8 Total prevalence of antibiotic resistance among *C. jejuni* vs. *C. coli* isolates in turkeys

3. Longitudinal trends in fecal prevalence and antibiotic resistance of *C. coli* from turkeys and swine, produced in close proximity

3.1 ABSTRACT

Campylobacter coli typically colonizes swine but has also been isolated from poultry, especially turkeys. In eastern North Carolina, a region that makes a major contribution to both turkey and swine production in the United States, turkey and swine farms commonly are in close proximity of one another, raising the possibility of transfer of *C. coli* between the respective animals. However, limited information is available on prevalence and antimicrobial resistance of *C. coli* from turkeys and swine in this region.

In this study, we investigated prevalence and antimicrobial susceptibility (to tetracycline, streptomycin, erythromycin, kanamycin, nalidixic acid, and ciprofloxacin) of *C. coli* isolates from fecal samples of turkeys and swine (n= 2960 total fecal samples) from 15 paired turkey/swine operations in this region. *C. coli* was isolated at high prevalence (56-95%) from all swine herds, and at varying prevalence (0-67%) from the corresponding turkey flocks. With exception of resistance to tetracycline, which was highly prevalent regardless of source, significant host-associated differences were found in the prevalence of resistance to all other tested antibiotics. Resistance to erythromycin and to streptomycin was significantly more prevalent in *C. coli* from swine than in isolates from turkeys ($p<0.0001$). In contrast, resistance to kanamycin was more frequent in *C. coli* from turkeys ($p<0.0001$), and resistance to nalidixic acid and ciprofloxacin was encountered in 78% of the turkey-derived *C. coli*, but not among any of the 1108 isolates from swine. These findings suggest that *C. coli* from these different hosts varied significantly in prevalence of resistance to certain antibiotics, even when production

operations were in close proximity to each other, and samplings were contemporaneous. Such differences in prevalence of antibiotic resistance may arise in response to differential antibiotic exposure regimens in different production systems, but may also indicate that sharing of *C. coli* strains between these alternative hosts is limited, possibly due to colonization by host-adapted strains.

3.2 INTRODUCTION

Campylobacter coli is second only to *C. jejuni* in implication to human campylobacteriosis, which currently is the leading foodborne illness of bacterial etiology in the United States and other industrialized nations (Friedmann et al, 2000). *C. coli* has long been recognized for its propensity to colonize swine (Aarestrup et al, 1997; Saenz et al, 2000; van Looveren et al, 2001; Avrain et al, 2004; Gebreyes et al, 2005), but has also been isolated from poultry. In recent studies in the United States, a significant portion of the thermophilic *Campylobacter* isolates from commercial turkeys pre-harvest or immediately after slaughter were *C. coli* (Logue et al, 2003; Smith et al, 2004; Lee et al, 2005; Luangtongkum et al, 2006). Resistance to certain antibiotics, especially erythromycin and the fluoroquinolones, has been found to be frequently more prevalent in *C. coli* than *C. jejuni* (Ge et al, 2003; Luber et al, 2003; Bae et al, 2005; Inglis et al, 2006; Luangtongkum et al, 2006).

North Carolina is a major contributor to turkey as well as swine production in the United States. Much of the turkey and swine production is concentrated in the eastern part of the state, with turkey and swine farms frequently in close proximity to each other. Major turkey companies (integrators) also have extensive involvement in swine production. In order to maximize family farm income in this largely rural region,

growers frequently produce both turkeys and swine. Such circumstances may enhance the likelihood that zoonotic organisms may transfer between turkeys and swine. In the case of *Campylobacter*, this is especially conceivable for *C. coli*, which has a propensity for swine but has also been frequently isolated from commercial turkey flocks in this region, and elsewhere (Smith et al, 2004; Lee et al, 2005; Wesley et al, 2005; Luangtongkum et al, 2006).

The intertwined turkey and swine production systems in eastern North Carolina presents opportunities for comparative investigations of prevalence and antimicrobial susceptibility profiles of *C. coli* from these two types of farm animals, and for investigations of transfer of *C. coli* between turkeys and swine. Prevalence of antimicrobial resistance may vary markedly among *C. coli*, depending on the host animal. For instance, a study in Denmark demonstrated that *C. coli* isolates from swine were more frequently resistant to erythromycin, enrofloxacin, and streptomycin than *C. coli* from broilers (Aarestrup et al, 1997). Comparable data on antimicrobial susceptibility profiles of *C. coli* from poultry and swine in the United States have been lacking.

One of the objectives of this study was to determine longitudinal trends in fecal prevalence of *C. coli* from commercial turkey and swine operations, surveyed concurrently. A second objective was to determine the impact of host animal (turkeys vs. swine) on prevalence of resistance of *C. coli* to selected antimicrobial agents.

3.3 MATERIALS AND METHODS

3.3.1 Paired-farm operations. Turkey and finishing swine farms were located in eastern North Carolina, under the operation of a single integrator. The framework of turkey production was previously described (Chapter 2). Swine production operates on an "all

in-all out" system, as does turkey production. Depending on the size of the farm operation, some farms had two finishing houses, while others had several houses. The pigs transitioned from the nursery to the finishing stage at approximately six weeks, and remained at the finishing farm until they reached market weight (~20 weeks).

3.3.2 Sampling scheme and bacterial isolation. A total of eight paired-farm operations were surveyed (twice in all but one case) from October 2003 to October 2005. The actual sampling scheme and procedures used to isolate *Campylobacter* from the turkey fecal samples were previously described (Chapter 2). Swine fecal samples were collected during the same visit to the turkey houses, when the turkeys were 5-6, 7-8, 10-11, and 12-14 weeks of age. Twelve fecal samples were collected from one finishing house during the first survey period, and the sample size increased to 20 samples during the second survey. In most cases, one sample was collected per pen as a representative sample (two samples collected from different corners of the pen if house had fewer pens), considering 10 to 12 pigs were confined to one pen and samples were commonly mixed or trampled upon. To randomize the sample collection, the pens were sampled in a "zig-zag" fashion. Collections were taken from two finishing houses, totaling 40 swine fecal samples per visit. Once the samples were collected, they were taken back to the laboratory for processing. *Campylobacter* isolations from the swine fecal samples were by direct plating, as done for the turkey fecal samples, and described before (Smith et al, 2004).

3.3.3 Antibiotic susceptibility testing, DNA extractions and species differentiation. Antibiotic susceptibility, DNA extractions, and species differentiation were performed in the same manner for the swine-derived *Campylobacter* isolates, as described for the turkey isolates (Chapter 2).

3.4 RESULTS

3.4.1 Prevalence of total *Campylobacter* and *C. coli* in turkeys and swine. A total of 1448 swine fecal samples were collected from the eight surveyed farms, and 1116 of the 1448 (77%) samples were positive for *Campylobacter* (Fig. 3.1). Of the 1116 *Campylobacter*-positive samples from swine, 1108 (99%) yielded *C. coli* (Fig. 3.2). *C. jejuni* was only isolated from only one sample. Seven samples yielded *Campylobacter* isolates that were negative with both the *C. jejuni* and the *C. coli*-specific PCR primers that we employed, and thus appeared to represent members of other *Campylobacter* species. Both *C. jejuni* and *C. coli* were isolated from turkey samples. Of the 1310 *Campylobacter*-positive samples from turkeys, *C. jejuni* was isolated from 781 (60%), and *C. coli* from the remaining 529 (40%). The prevalence of total campylobacters, *C. jejuni* and *C. coli*, in fecal samples from each of the eight paired farms (eight turkey and eight swine farms) is summarized in Table 3.1. The prevalence data of *Campylobacter* in these turkey farms have been described in detail elsewhere (Chapter 2), and have been included here to facilitate the comparisons with the data from the swine farms.

C. coli was frequently isolated from all 15 swine herds, with prevalence among different herds ranging from 56 to 95%. Of the 15 turkey flocks, 12 also had significant prevalence of *C. coli* (30-67%). Two flocks (T4B and T7B) had low prevalence of the organism, and *C. coli* was not detected in one flock (T4A) (Table 3.1). High prevalence of *C. coli* in a specific swine herd was not always accompanied by high prevalence in the samples from the paired turkey flock. For instance, *C. coli* was recovered from the majority of the swine samples from farms S4 and S7, but samples from the corresponding

turkey farms (T4B, T7A, T7B; samples from T4A were largely *Campylobacter*-negative) yielded mostly *C. jejuni* (Table 3.1).

Comparisons of *C. coli* recovery from the two different surveys of the same farm (indicated by A and B in Table 3.1) revealed fluctuations (typically a decreasing trend) in certain turkey and swine farms. For turkey farms T5 and T6, 46% of the samples yielded *C. coli* during the first survey period. However, the frequency of colonization declined to 34% and 30% for T5 and T6, respectively, during the second period. Farm T7 experienced an even greater decline in *C. coli* colonization between survey periods, dropping from 19% to 2%. A similar, but less marked trend, was noted in *C. coli* recovery from the corresponding swine farms (Table 3.1).

3.4.2 Impact of age on turkey- and swine-derived *C. coli* isolates. Prevalence of *C. coli* in the swine samples was constant throughout the survey period of the herds. In contrast, prevalence of *C. coli* in turkeys was higher in the younger birds (5-6 weeks of age) and declined during transition to grow-out, remaining constant from then on (Table 3.2 and Fig. 3.3).

3.4.3 Seasonal impact on colonization of *C. coli* in turkeys and swine. The prevalence of *C. coli* in turkeys and swine was similar for both seasonal periods: winter/spring and summer/fall. Although the number of summer/fall samples was almost double that of winter/spring samples, the overall rates of recovery were similar for both seasonal periods (Table 3.3).

3.4.4 Antibiotic resistance of *C. coli* derived from turkey and swine isolates. Resistance to tetracycline was highly prevalent in both turkey and swine isolates (90 and 99%, respectively) (Table 3.4 and Fig. 3.4). However, prevalence of resistance to the

other tested antibiotics varied noticeably depending on the host (Table 3.4 and Fig. 3.4). *C. coli* from swine were more frequently resistant to erythromycin (82%) and to streptomycin (58%) than *C. coli* from turkeys (27% and 31%, respectively) ($p<0.0001$). On the other hand, *C. coli* from turkeys were more frequently resistant to kanamycin (63%) than *C. coli* from swine (26%) ($p<0.0001$), and resistance to nalidixic acid and ciprofloxacin was detected in 78% of *C. coli* from turkeys, but not among any of the 1108 isolates of *C. coli* from swine. In contrast to turkey-derived *C. coli*, for which prevalence of resistance to certain antibiotics varied with age of the birds, such temporal trends were not detected in *C. coli* from swine, with any of the tested antimicrobials (Table 3.4 and Fig. 3.4).

Partly due to the absence of fluoroquinolone resistance among swine-derived *C. coli*, the antimicrobial susceptibility profiles prevalent among this population were different than those prevalent among the turkey-derived *C. coli*. Thus, TSEKQ, a multi-drug resistance profile, was prevalent among turkey isolates, but not detected among swine isolates, for which TSE and TE were the most prevalent profiles (Table 3.5).

3.4.5 Prevalence of antibiotic resistance in *C. coli* isolates from different surveys of the same farm. Farm T8 was chosen to examine possible differences in antibiotic resistance prevalence of *C. coli* isolated from two different survey periods. In comparison to T8A, slightly higher levels of resistance to tetracycline, kanamycin, and nalidixic acid and ciprofloxacin were observed in T8B. No significant differences were observed in the swine isolates between the two survey periods (data not shown).

3.5 DISCUSSION

In this study, *C. coli* was isolated with significant frequency (>20% of the samples) from 12 of the 15 turkey flocks that were surveyed. Prevalence exceeded 50% in four of the flocks. In contrast, all 15 corresponding swine farms had high prevalence of *C. coli*, (> 70% in 11, and 56-65% in the other four). Furthermore, all three of the turkey flocks with low prevalence (0-11%) of *C. coli* were in close proximity with swine herds with high prevalence (78-95%) of the organism. These findings suggest that *C. coli* may colonize turkeys independently from swine, and that prevalence levels in a swine farm cannot be used to predict levels in a nearby turkey farm. These findings also suggest that *C. coli*, well-known for its ability to colonize swine, may not transfer frequently from the swine to the turkeys, even when the farm operations were in close proximity to each other, as was the case in this study, and the common grower tended to both operations.

Prevalence of resistance to several structurally unrelated antibiotics was markedly different between turkey- and swine-derived *C. coli*. Streptomycin and erythromycin resistance was observed more frequently in *C. coli* from swine, whereas isolates from turkeys were noticeably more frequently resistant to kanamycin. The most pronounced difference was in regard to fluoroquinolones, resistance to which was encountered frequently among *C. coli* from turkeys, but was not detected in any of the 1108 isolates from swine.

High prevalence (78%) of streptomycin resistance among swine-derived *C. coli* was also observed in a recent study in Switzerland (Schuppers et al, 2005). Furthermore,

Aarestrup et al (1997) reported that resistance to streptomycin was more frequent among *C. coli* isolates from swine (48%) than from broilers (6%) or humans (0%).

In this study, erythromycin resistance was noticeably more prevalent in *C. coli* from swine (82%) than turkeys (27%). Reports of high rates of erythromycin resistance in *C. coli* from swine (Aarestrup et al, 1997; Saenz et al, 2000; van Looveren et al, 2001; Avrain et al, 2004), agree with this study's findings. However, two recent studies reported significantly lower frequencies of erythromycin resistance (ca. 40% and 19 %) in *C. coli* from swine (Gebreyes et al, 2005; Schuppers et al, 2005). Antimicrobial regimes in different farms may at least partially account for the observed differences in prevalence among different studies.

Prevalence of resistance to erythromycin among turkey-derived *C. coli* has varied in different studies. Low prevalence (0% to 12.5%) was described in a study in Germany in which isolates from 1991 and 2001 were compared (Luber et al, 2003). Conversely, a high prevalence (80%), also higher than what was observed in the current study, was noted among conventionally raised turkeys from 10 flocks in Iowa, sampled after slaughter during August 2000 to November 2002, at the processing plant (Luangtongkum et al, 2006). Only 18% of samples from birds close to marketing in our study were resistant to erythromycin (higher prevalence was in samples from younger birds). It is noteworthy that in an earlier study of three turkey flocks in eastern North Carolina we also had found noticeably higher frequency of *C. coli* that harbored multi-drug resistance, including resistance to erythromycin (Lee et al, 2005). Such differences may again reflect different antibiotic use in the farms that were surveyed, therapeutically or for

growth promotion. Further studies will be needed to identify possible temporal trends towards decreasing prevalence of resistance to erythromycin in *C. coli* from turkeys.

Kanamycin (63%) and fluoroquinolone (78%) resistance was significantly higher in *C. coli* from turkeys. Younger birds were more resistant to both agents than older birds, suggesting possible administration of these antibiotics (or a structurally related agent) during the earlier stages of production. High rates of fluoroquinolone resistance in *C. coli* from turkeys after slaughter or from turkey meats have been reported in several studies (Saenz et al, 2000; van Looveren et al, 2001; Ge et al, 2003; Luber et al, 2003; Luangtongkum et al, 2006), and in our previous surveys we detected high prevalence in turkeys pre-harvest (Lee et al, 2005). Even though fluoroquinolone resistance was not detected among any of the 1108 isolates of swine-derived *C. coli* that we tested, several studies from Europe have detected fluoroquinolone resistance (ranging from 17 to 100%) among *C. coli* from swine (Aarestrup et al, 1997; Saenz et al, 2000; van Looveren et al, 2001; Avrain et al, 2004; Schuppers et al, 2005). In a recent study of *C. coli* from swine (sampled after slaughter) in the United States, 27 of the isolates were found to be fluoroquinolone-resistant, and frequently were resistant to nalidixic acid but susceptible to ciprofloxacin (Gebreyes et al, 2005).

The reasons for these differences in prevalence of fluoroquinolone resistance of *C. coli* from swine remain unclear, but may reflect antibiotic use regimes and animal husbandry practices that would likely differ in animals/herds surveyed in different studies. Communication with the company veterinarians indicated that fluoroquinolones were never in use in the swine herds, whereas fluoroquinolones (Baytril®) were still permitted for use in turkeys during the duration of the study. On the other hand, certain

strains of *C. jejuni* have been shown to maintain resistance to fluoroquinolones even when the antibiotics are not in use, reflecting greater fitness of the fluoroquinolone-resistant organisms (Luo et al, 2005).

If that would also be the case for *C. coli*, the observed complete absence of fluoroquinolone resistance among *C. coli* from swine may suggest that *C. coli* harboring such resistance were not frequently transferred from turkeys to swine, even in the intertwined turkey-swine production systems investigated in this study. Considering the close proximity between the farms, and the presence of common growers, one might expect that *C. coli* could be transferred between these hosts, and that fluoroquinolone-resistant strains from turkeys could be introduced into a population in which they would not commonly be found. Alternative approaches to detect strains that may transfer rarely may need to be employed, and such approaches (e.g. isolation of bacteria on selective media containing nalidixic acid or ciprofloxacin) as described by Nannapaneni et al (2005) are being planned in our laboratory. Even though antibiotic regimes may select certain strains of *C. coli* in turkeys versus swine, it is also likely that these animals may be colonized by host-adapted strains which are genetically distinct, regardless of antibiotic resistance. Recent studies employing multiple locus sequence typing indeed revealed several turkey-associated and swine-associated strain types (Miller et al, 2005). Further studies are needed to determine strain subtypes of the *C. coli* isolates in this study, and determine possible relatedness between turkey- and swine-derived strains.

In summary, our findings revealed significant differences in prevalence and resistance to certain antibiotics between turkey- and swine-derived *C. coli* isolated from intertwined turkey/swine production systems. Considering the fact that antibiotics with

marked host-associated prevalence included those used for treatment of human illness (fluoroquinolones and erythromycin), the findings would contribute to further evaluation of the human public health risk posed by zoonotic *C. coli*. Further studies are needed to determine continuing trends in prevalence and antibiotic resistance, and to elucidate strain relatedness (and possible strain transfer) of *C. coli* from these different animal hosts.

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Table 3.1 Prevalence of *Campylobacter*, *C. jejuni*, and *C. coli* in turkeys and swine among paired-farm production facilities

Farm[1]	Survey Period	*Campylobacter*	*C. jejuni*	*C. coli*
T1A	Oct 2003-Dec 2003	36/36 (100)	13/36 (36)	23/36 (64)
S1A		35/36 (97)	0/36 (0)	35/36 (97)
T1B	June 2004-Aug 2004	84/92 (91)	48/92 (52)	36/92 (39)
S1B		70/92 (76)	0/92 (0)	70/92 (76)
T2	Nov 2003-Dec 2003	35/36 (97)	11/36 (31)	24/36 (67)
S2		34/36 (94)	0/36 (0)	32/36 (89)[2]
T3A	Feb 2004-March 2004	47/48 (98)	22/48 (46)	25/48 (52)
S3A		27/48 (56)	0/48 (0)	27/48 (56)
T3B	June 2005-Aug 2005	143/160 (89)	55/160 (34)	88/160 (65)
S3B		82/140 (59)	0/140 (0)	82/140 (59)
T4A	March 2004-April 2004	1/48 (2)	1/48 (2)	0/48 (ND)
S4A		35/36 (97)	0/36 (0)	35/36 (97)
T4B	July 2004-Aug 2004	35/36 (97)	31/36 (86)	4/36 (11)
S4B		34/36 (94)	0/36 (0)	34/36 (94)
T5A	March 2004-May 2004	48/48 (100)	26/48 (54)	22/48 (46)
S5A		34/36 (94)	0/36 (0)	34/36 (94)
T5B	Aug 2005-Oct 2005	130/160 (81)	75/160 (47)	55/160 (34)
S5B		134/160 (84)	0/160 (0)	134/160 (84)
T6A	March 2004-May 2004	48/48 (100)	26/48 (54)	22/48 (46)
S6A		41/48 (85)	0/48 (0)	41/48 (85)
T6B	Aug 2005-Oct 2005	99/160 (62)	51/160 (32)	48/160 (30)
S6B		124/160 (78)	1/160 (<1)	120/160 (75)[3]
T7A	Sep 2004-Nov 2004	157/160 (98)	121/160 (76)	36/160 (23)
S7A		138/160 (86)	0/160 (0)	138/160 (86)
T7B	Jan 2005-March 2005	138/160 (86)	134/160 (84)	4/160 (3)
S7B		126/160 (79)	0/160 (0)	124/160 (78)[4]
T8A	Oct 2004-Nov 2004	150/160 (94)	75/160 (46)	75/160 (47)
S8A		98/140 (70)	0/140 (0)	98/140 (70)
T8B	Dec 2004-Feb 2005	160/160 (100)	86/160 (54)	74/160 (46)
S8B		104/160 (65)	0/160 (0)	104/160 (65)

1 Paired farms have the same number, and letters (A,B) indicate repeated surveys at the same production facilities, during the indicated time periods

2, 3, 4 Indicate undesignated species for swine isolates

Table 3.2 Prevalence of turkey- and swine-derived *C. coli* isolates for the four sampling time points

Sampling Time Points	Source	Total Samples	*C. coli*
1	Turkey	282	153/282 (54)[1]
	Swine	377	298/377 (79)
2	Turkey	358	136/358 (38)
	Swine	374	271/374 (72)
3	Turkey	365	122/365 (33)
	Swine	364	284/364 (78)
4	Turkey	356	118/356 (31)
	Swine	336	255/336 (76)

1 Percentages indicated in parentheses

Table 3.3 Seasonal impact on recovery of *C. coli* from turkey and swine fecal samples

Source	***Campylobacter* spp.**	**Winter/Spring Turkeys (n=548) Swine (n=526)**	**Summer/Fall Turkeys (n=965) Swine (n=925)**
Turkeys	*C. coli*	171 (31)[1]	358 (37)[1]
Swine	*C. coli*	397 (75)	711 (77)

n indicates total number of turkey and swine fecal samples collected per season

1 Percentages are indicated in parentheses

Table 3.4 Antibiotic resistance among turkey- and swine-derived *C. coli* isolates at different sampling times

		Resistance to				
Source	**Sampling Time[1]**	**Tet**	**Str**	**Em**	**Kan**	**Nal/Cipro**
Turkeys	1 (n=153)	143 (93)[2]	60 (39)	54 (35)	115 (75)	128 (84)
	2 (n=136)	122 (90)	39 (29)	36 (26)	87 (64)	121 (89)
	3 (n=122)	117 (96)	39 (32)	32 (24)	71 (58)	86 (70)
	4 (n=118)	110 (92)	27 (24)	22 (18)	60 (52)	80 (68)
	Total=529	492 (93)	165 (31)	144 (27)	333 (63)	415 (78)
Swine	1 (n=298)	291 (98)[2]	179 (60)	245 (82)	55 (18)	0 (0)
	2 (n=271)	260 (96)	153 (56)	218 (80)	72 (27)	0 (0)
	3 (n=284)	283 (99)	165 (58)	239 (84)	102 (36)	0 (0)
	4 (n=255)	252 (98)	142 (56)	207 (81)	55 (22)	0 (0)
	Total=1108	1086 (98)	639 (58)	909 (82)	284 (26)	0 (0)

1 Samples at sampling times 1, 2, 3, and 4 were obtained when turkeys were 5-6, 7-8, 10-11, and 12-14 weeks of age, respectively; n indicates total number of *C. coli* isolates from the indicated sampling times.

2 Percentages are indicated in parentheses

Table 3.5 Antibiotic resistance profiles in *C. coli* isolates from turkeys and swine

AB^R Profile	Turkeys		Swine	
No Resistance	1	(0.2%)[1]	4	(0.4%)
E	0	(0%)	2	(0.3%)
K	1	(0.2%)	0	(0%)
Q	30	(6%)	0	(0%)
S	0	(0%)	8	(0.7%)
T	35	(7%)	44	(4%)
EK	0	(0%)	1	(0.1%)
KQ	3	(0.6%)	0	(0%)
SE	0	(0%)	7	(0.6%)
SQ	2	(0.4%)	0	(0%)
TE	2	(0.4%)	275	(25%)
TK	58	(11%)	24	(2%)
TQ	109	(21%)	0	(0%)
TS	0	(0%)	84	(8%)
SKQ	0	(0%)	0	(0%)
TEK	1	(0.2%)	119	(11%)
TEQ	1	(0.2%)	0	(0%)
TKQ	107	(20%)	0	(0%)
TSE	0	(0%)	400	(36%)
TSK	7	(1%)	35	(3%)
TSQ	16	(3%)	0	(0%)
TEKQ	16	(3%)	0	(0%)
TSEK	9	(2%)	105	(9%)
TSKQ	16	(3%)	0	(0%)
TSEKQ	115	(22%)	0	(0%)

1 Percentages are indicated in parentheses

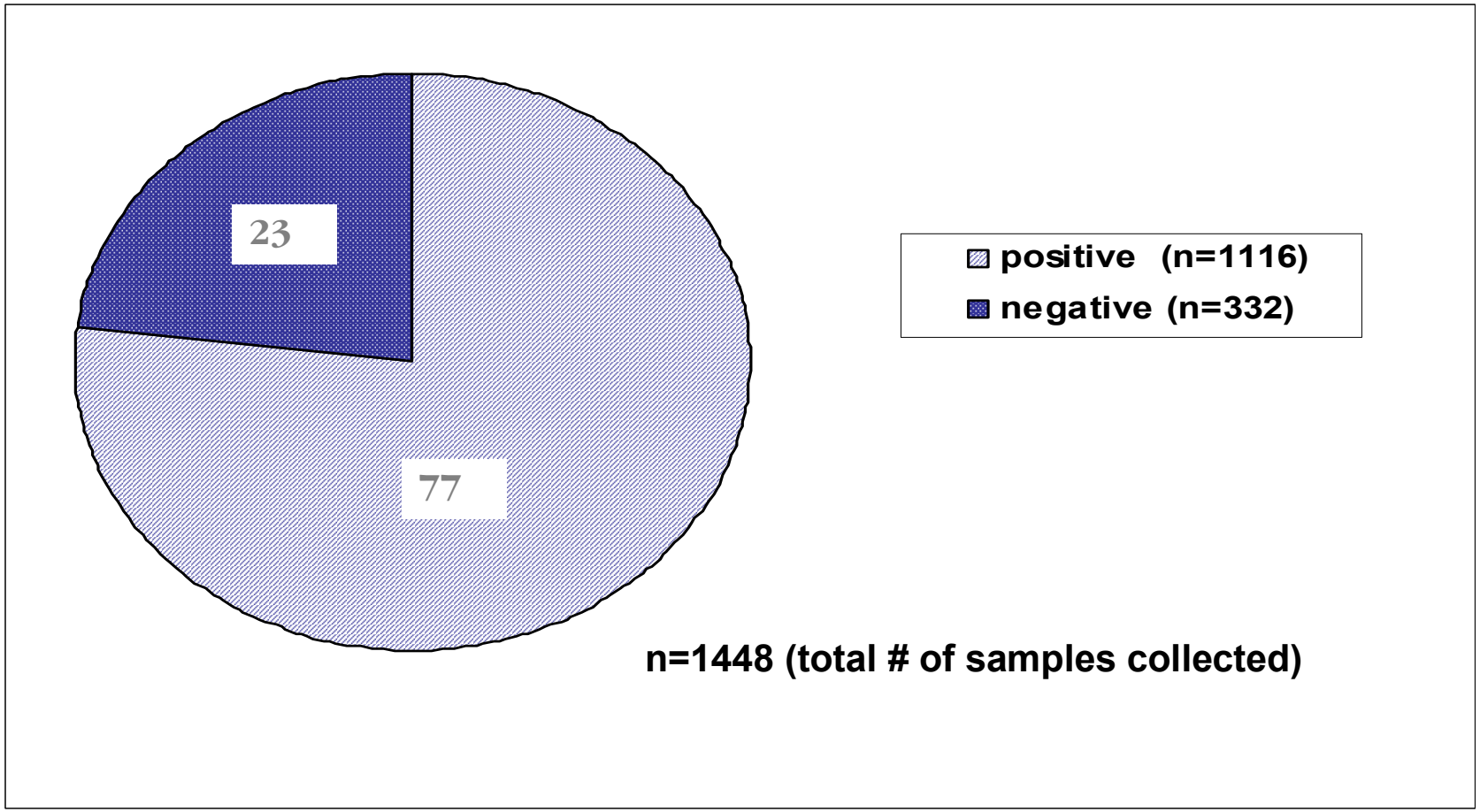

Figure 3.1 Recovery of *Campylobacter* from swine fecal samples

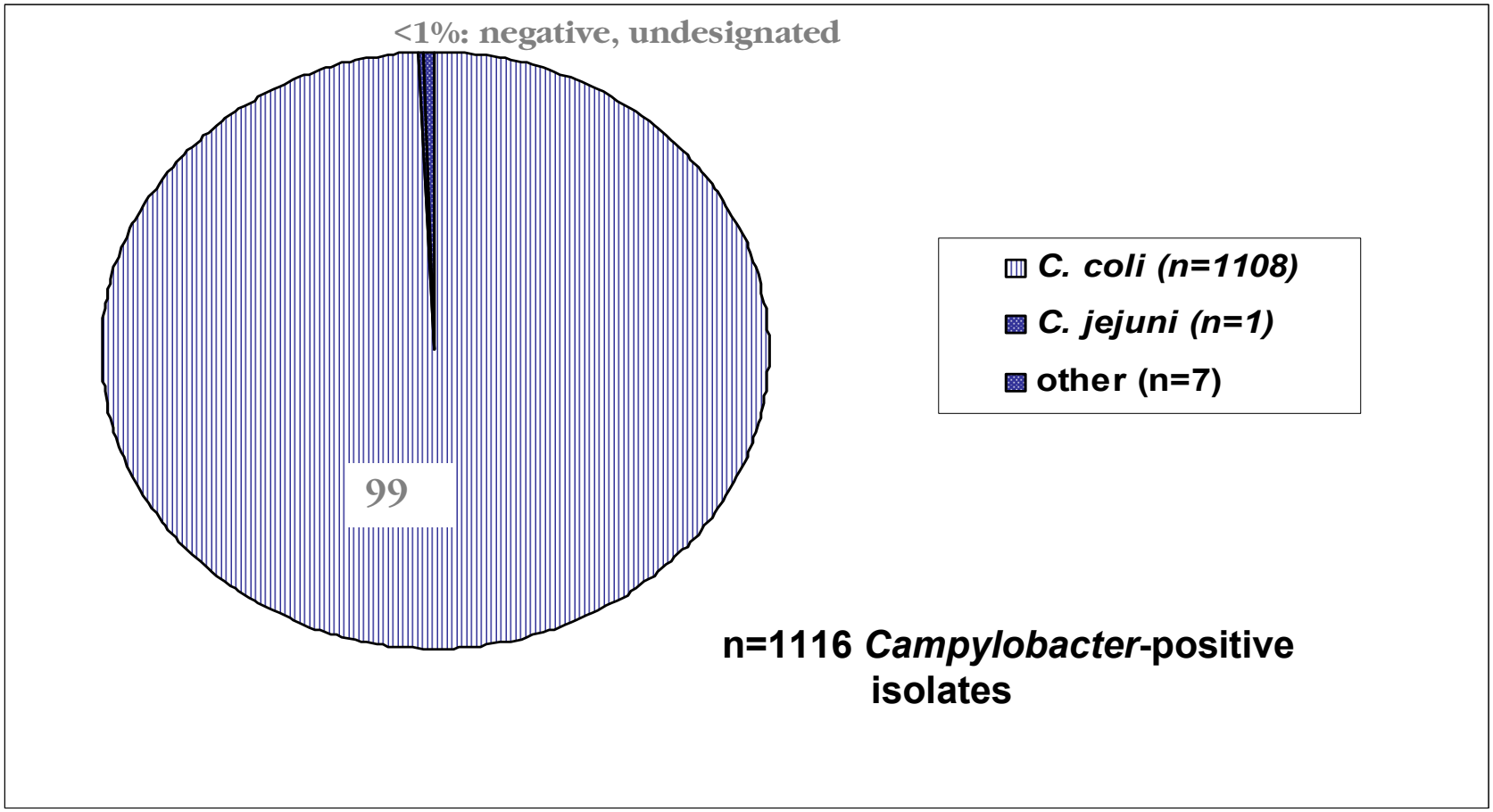

Figure 3.2 Prevalence of *C. jejuni* vs. *C. coli* among herds

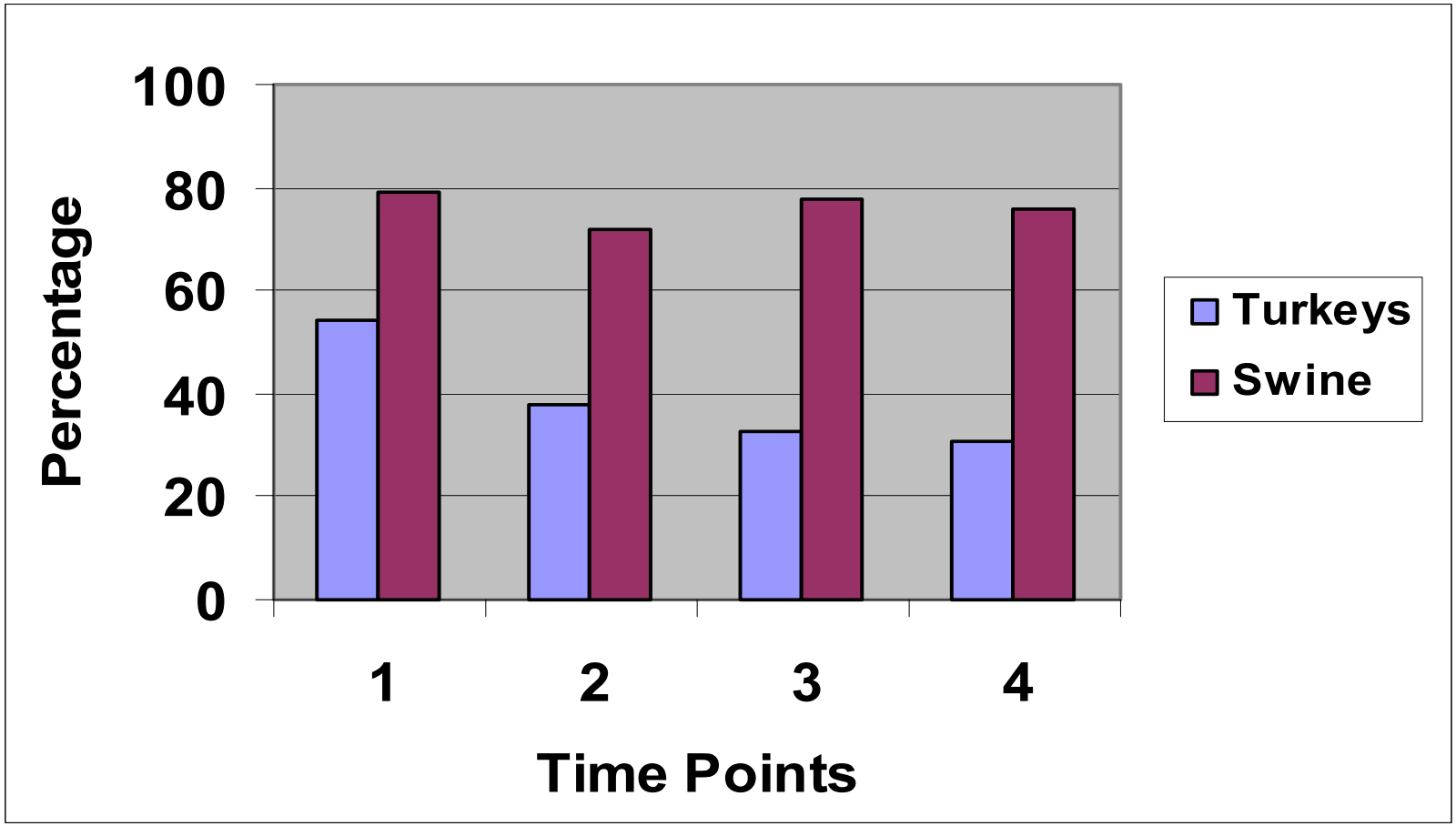

Figure 3.3 Prevalence of *C. coli* in turkeys and swine at four time points

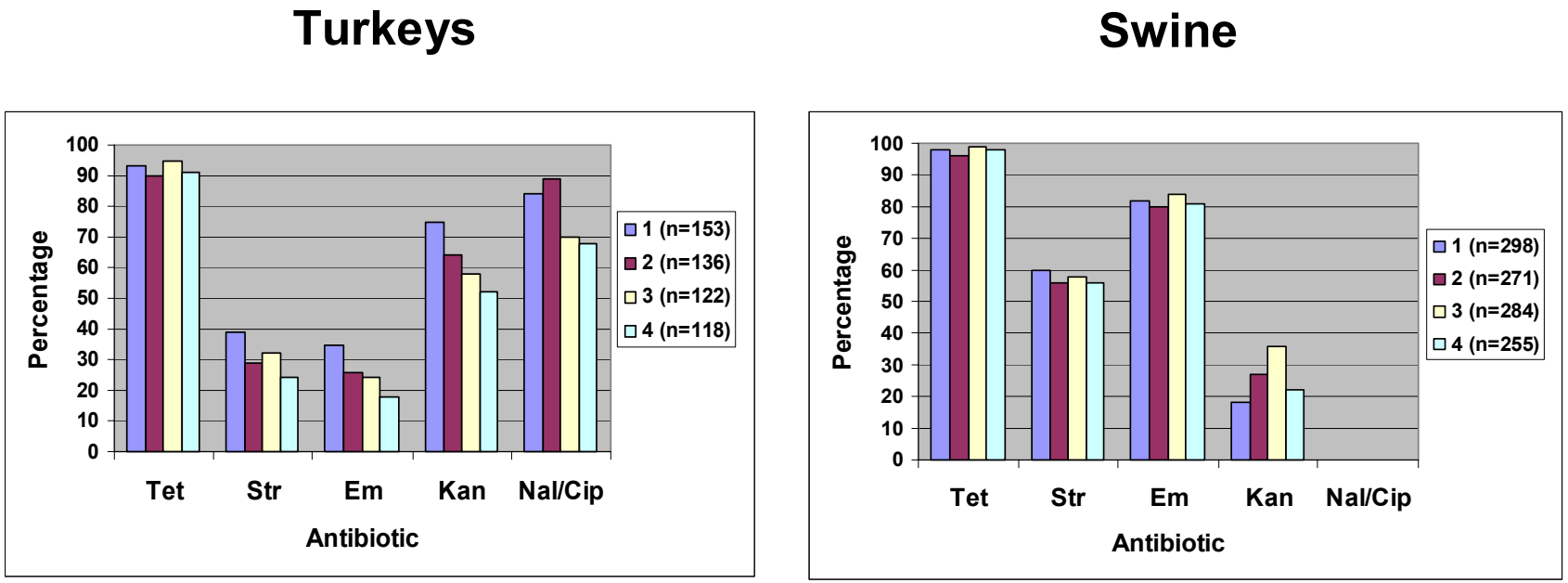

Figure 3.4 Antibiotic resistance in *C. coli* from turkeys and swine

4. Genetic relationships between *C. coli* strains from turkeys and swine determined by molecular subtyping methods

4.1 ABSTRACT

Campylobacter coli has a well-documented propensity for colonizing swine, but has also been found to colonize poultry, especially turkeys. In certain regions of the United States, turkeys and swine are grown in close proximity (paired operations), suggesting the potential for transfer of *C. coli* between the respective animal hosts. In this study, we employed multilocus sequencing (MLST), pulsed-field gel electrophoresis (PFGE) with two enzymes (*Sma*I and *Kpn*I), and *fla*-typing to characterize *C. coli* from 15 different paired turkey-swine operations. Several distinct strain clusters were identified among isolates from turkeys and swine, suggesting that the corresponding clonal groups are stable in the corresponding hosts. However, the overall strain subtyping data failed to identify strain types that were shared between *C. coli* isolates from turkeys and swine, suggesting that transfer of the organism between the corresponding operations may be infrequent, or may take place under special conditions.

4.2 INTRODUCTION

Campylobacter is the leading bacterial agent of human diarrheal illness in the United States and other industrialized nations, with *Campylobacter jejuni* and *C. coli* being the species most frequently involved in foodborne cases of illness. *C. jejuni* and *coli* have the propensity to infect an array of animals, particularly agricultural livestock. Meat animals are frequently colonized with *Campylobacter*, up to 100% of the population. Poultry are predominantly colonized with *C. jejuni*, whereas swine are the

primary reservoir of *C. coli*. From an epidemiological standpoint, it has been difficult to trace the organism through the food supply chain to identify it as a source of infection.

Subtyping (or genotyping) methods are molecular analyses of the bacterial genome to characterize different strains within a species. Typically, a species will consist of a multitude of strains, and particular strains are involved in food-related cases. Molecular subtyping makes it possible to identify those strains linked to human infection. Likewise, subtyping can determine genetic relatedness between strains from different sources. Different subtyping tools characterize different regions of the bacterial genome. Sequence polymorphisms in the flagellin gene *flaA* are the basis for *fla*-typing, whereas the entire genome is the basis for pulsed-field gel electrophoresis (PFGE) typing. The discriminatory power of PFGE (or DNA fingerprinting) makes it a superior analytical tool for characterizing bacterial strains. Chromosomal DNA is embedded in agarose plugs and cleaved with restriction enzymes that cut DNA infrequently, producing large fragments that are separated via electrophoresis (Wassenaar and Newell, 2000). Multilocus sequence typing (MLST) provides unambiguous and highly portable results, which other genotyping methods cannot attain. Several housekeeping genes are sequenced for each strain within a species and the sequences at each locus are assigned an allele number. An allelic profile of a strain is based on the combination of the different alleles and each different profile is assigned a sequence type (ST) that describes the strain (Aanensen and Spratt, 2005).

In the current study, molecular subtyping analyses (MLST, PFGE, *fla*-typing) were used to characterize *C. coli* strains from turkeys and swine produced in close proximity, representing eight paired-farm operations located in eastern North Carolina.

These analyses determined genetic relatedness and established any putative relationships between *C. coli* strains from different animal hosts.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial samples used for molecular subtyping. Bacteria isolated from fecal samples collected from turkey and swine houses (Chapters 2 and 3) were used for genotypic characterization of *C. coli* strains from the two animal hosts. Isolates to be typed were selected according to host, date of isolation, and antibiotic resistance profile.

4.3.2 Multilocus sequence typing (MLST). Chromosomal DNA samples from 99 isolates were prepared with the Qiagen DNeasy Kit (Qiagen) and shipped for sequence typing to the USDA-ARS-WRRC laboratory located in Albany, California headed by Robert Mandrell. Sequencing was performed as described by Miller et al (2006), using seven primer sets for amplification, and allelic sequences obtained in the study were compared to those in the *C. jejuni/C. coli* MLST database (http://pubmlst.org/campylobacter/) (Jolley et al, 2004). This allowed for the determination of sequence types (STs), and new ST designations were provided by the MLST database. The data were incorporated into Bionumerics (version 3.5; Applied Maths) and a minimum spanning tree was created to demonstrate the genetic relatedness among isolates based on their sequence types (STs). ST diversity was determined as the ration of total number of STs in a given population over the total number of MLST-characterized isolates in the population. The maximum value for diversity was 1.0 (one ST per isolate).

4.3.3 Pulsed-field gel electrophoresis (PFGE). A total of 426 isolates (300 from swine and 126 from turkeys) were typed by PFGE. PFGE analysis of bacterial isolates using *Sma*I was conducted as described by Ribot et al (2001). *Kpn*I was used as a secondary

enzyme for PFGE analysis. Bionumerics (version 3.5; Applied Maths) was used to analyze PFGE pattern relatedness among different isolates. A dendogram UPGMA analysis was constructed, with 2.0% optimization and band position tolerance. PFGE type diversity was determined as the ration of total number of PFGE types in a given population over the total number of PFGE-characterized isolates in the population. The maximum value for diversity was 1.0 (one PFGE type per isolate).

4.3.4 *fla*-typing. Chromosomal DNA was obtained using the Qiagen DNeasy kit (Qiagen) and amplification of *flaA* was achieved with a *fla*-specific primer set (Fla forward and Fla reverse) (Operon; Chicago, IL) as described (Smith et al, 2004). The PCR product was digested by the restriction enzyme *Dde*I (New England Biolabs; Waverly, MA). Bionumerics software (version 3.5; Applied Maths) was employed to analyze relatedness among fla types. A dendogram UPGMA analysis was constructed, with 2.0% optimization and band position tolerance. Diversity of *fla* types was determined as the ration of total number of *fla* types in a given population over the total number of *fla*-typing-characterized isolates in the population. The maximum value for diversity was 1.0 (one *fla* type per isolate).

4.4 RESULTS

4.4.1 Characterization of *C. coli* strains by MLST. On the basis of MLST, 20 STs were identified among the 37 turkey-derived *C. coli* isolates (diversity index 0.47) and 29 STs were identified among the 62 isolates from swine (diversity index 0.47). MLST identified two and seven new STs for turkeys and swine, respectively. Of these STs, two from turkey-derived isolates (1833, 1835) and seven from swine (1829, 1830, 1831,

1832, 1834, 1836, 1837) isolates were new, not being described before in the *Campylobacter* MLST database.

In the minimum spanning tree constructed on the basis of the ST data, 28 of the 29 STs from swine were clustered in swine-associated groups; only ST-1164, identified in one swine isolate, was grouped with STs identified in turkey isolates, being derived from the turkey-associated ST-889. All of the swine-associated STs appeared to derive from a central ST (ST-828), which was also detected in one of the swine isolates characterized in this study (Fig. 4.1). STs of turkey isolates were distributed among three groups. Two of the groups (one including STs 1126, 1154, and 1163, and the other including STs 1017, 1101, 889, 1149, and 1160) appeared to derive from swine-associated STs (ST-1055 and ST-828, respectively). In contrast, the STs in the third group (designated cluster II in Fig. 4.1) lacked noticeable relatedness to swine-associated STs. These cluster II strains also appeared to be distantly related to other turkey-derived strains, with more than three allele differences from the latter (Fig. 4.1). Even though some of the turkey-associated STs (e.g. ST-1017 and ST-1126) could be derived from swine STs by one (ST-828) or two (ST-1055) allelic changes, STs found in both turkey- and swine-derived isolates were not detected (Fig. 4.1).

4.4.2 PFGE analysis of *C. coli* isolates from turkeys and swine using *Sma*I and *Kpn*I.

A total of 426 *C. coli* isolates (126 from turkeys and 300 from swine) were analyzed by PFGE with *Sma*I. A total of 53 PFGE types were identified among the 126 turkey isolates, and 131 PFGE types were identified among swine-derived isolates, leading to PFGE diversity indices of 0.42 and 0.44, respectively. Forty turkey-associated strain types were found in one or two isolates. When two isolates shared the same profile, they

invariably derived from the same flock, at either the same or different time points. *Sma*I analysis detected 13 strain types in clusters containing three or more turkey isolates, with the majority including three to four isolates from the same farm and flock, including those from different time points. Three large clusters (two with six isolates and the third had 12) of identical *Sma*I types were observed among the turkey strains. These three large clusters were the only ones that contained isolates from different farms. The two clusters containing six isolates each, derived from two and three farms, respectively. The largest cluster (12 isolates) included isolates from four different farms, and various time points.

A total of 300 swine isolates were typed with *Sma*I, detecting 101 types in one or two isolates and 30 clusters containing three or more isolates (data not shown). In most cases, clusters containing two isolates with indistinguishable patterns, derived from the same farm and herd (at the same or different time point). Of the 30 clusters consisting of three or more isolates, 10 included five or more isolates, and two of them were especially large (14 and 18 isolates, respectively). The cluster of 14 isolates included organisms from three different farms, including different herds (both survey periods) on farm S7. Likewise, the other large cluster of 18 isolates derived from four different farms, with the same strain being detected between survey periods of farm S4. PFGE types shared by turkey- and swine-derived *C. coli* were not detected, with the sole exception of strain 6085, a swine-derived isolate with a *Sma*I type that appeared to be indistinguishable from types of several isolates from turkeys.

The majority of the isolates (416/426) typed with *Sma*I were also examined by PFGE using *Kpn*I, including 124 isolates from turkeys and 292 from swine. A larger

number of strain types were observed with *Kpn*I: 80 among the 124 turkey isolates, and 142 among the 292 swine isolates, suggesting diversity indices of 0.65 and 0.49, respectively. Many strain types (71 and 119 from turkey- and swine-derived isolates, respectively) were encountered in only one or two isolates. In most cases, pairs of isolates with the same *Kpn*I type were derived from the same flock or herd, at either the same or different time point.

*Kpn*I analysis detected 9 and 24 types in clusters containing three or more turkey and swine isolates, respectively, with the majority of these clusters containing three to four isolates. For turkeys, two large clusters of isolates with identical types (six each), were derived from four and two farms, respectively. Large clusters of swine isolates consisted of five to eight isolates, with the largest containing 23 isolates. This largest cluster contained isolates from four different farms (at varying time points) and from different herds on farms S4 and S7.

A composite analysis (with both enzymes) revealed that some of the large clusters of closely related strains detected with either *Sma*I or *Kpn*I could also be identified when both enzymes were used. There was no detectable cluster of closely related types, on the basis of both enzymes, that contained strains from both turkeys and swine. Typically, clusters consisted of either turkey-derived or swine-derived strains. The swine-derived strain 6085, found to have a *Sm*aI type also found in turkeys isolates, had a clearly distinct *Kpn*I type and on the basis of the combined *Sma*I-*Kpn*I analysis was not grouped together with turkey-derived isolates.

4.4.3 *fla*-typing of *C. coli* strains. *fla*-typing of 445 isolates, including 129 from turkeys and 316 from swine, revealed 59 *fla* types in turkey-derived isolates and 67 among

isolates from swine, leading to diversity indices of 0.46 and 0.21, respectively. Host-associated trends noted in the PFGE analysis were similar to those for *fla*-typing. However, clusters containing *C. coli* strains from both animal hosts with indistinguishable *fla* patterns were identified. Even though a few strains from turkeys and swine shared common *fla* types, the majority did not, and the *fla*-based clusters consisted primarily of turkey-derived and swine-derived strains. A majority of the clusters consisting of isolates from both animal sources contained organisms from the same farm, at the same or different time points. One cluster (n= 7) in particular contained the same organism between different flocks on farm T1.

Forty-three *fla* types were observed in one or two turkey isolates, with pairs mostly deriving from the same flock, surveyed at the same time point. A total of 16 *fla* types were detected in clusters consisting of three or more isolates, with the majority of these having three to four isolates. Many of the clusters contained isolates from the same farm, including the same or various time points. Several large clusters of five to seven isolates were observed, with the two clusters of seven isolates deriving from five and four flocks, respectively. Each of these flocks represented a different farm, and was surveyed at different time points.

Among swine-derived isolates, 41 *fla* types were detected in only one or two isolates, with all pairs being derived from the same farm and herd, and the same time point (with the exception of one pair that differed only in time of sampling). Twenty-six clusters consisted of three to twelve isolates, with the majority having three to four isolates. The largest clusters contained 25 to 54 isolates, all including organisms deriving from a majority (either five or six) of the surveyed farms. The clusters containing 25 and

39 isolates of identical types included organisms from different herds on farms S7 and S4, respectively. The same organism was detected between survey periods (different herds) of farms S1, S4, and S7 in the clusters consisting of 34 and 54 isolates.

4.4.4 Agreement between MLST, PFGE and *fla* type data regarding the genetic relatedness of *C. coli* strains from turkeys and swine. Host-associated trends that were identified by MLST appear to correspond to those for PFGE, and many of the turkey-associated STs that consisted of two or more isolates had identical or closely related PFGE types. Comparing data from both MLST and PFGE revealed that turkey-associated STs in cluster II (Fig. 4.1) appeared to be closely related by PFGE (using both *Sma*I and *Kpn*I), and were clustered separately from other turkey-derived strains, with cluster I STs (data not shown). The largest of the turkey-derived clusters based on PFGE (n= 12) contained two cluster II STs (1150, 1487), which were closely related, as shown in Figure 4.1. Other turkey-associated ST clusters in cluster I (889, 1017, 1101, 1149, 1160 and 1126, 1154, 1163) were found to have similar PFGE types (data not shown). Such data suggested that certain STs may have been significantly more prevalent in our population than suggested by their prevalence among the relatively limited number of isolates that could be MLST-typed. For instance, one PFGE cluster included 12 turkey isolates with identical or closely related PFGE profiles. STs were determined for only three of the isolates in this cluster, and found to be ST-1150 (two isolates) and the closely related ST-1487 (one isolate). The similarity in PFGE patterns among the isolates; however, suggest the likelihood that the remaining isolates may also have these STs (from cluster II in Fig. 4.1).

Similar findings were observed for swine isolates. Clustering of swine isolates by MLST (Fig. 4.1) was similar to clusters observed by a *Sma*I-*Kpn*I composite analysis (data not shown). Six swine-associated clusters were identified by MLST (Fig. 4.1), and many of these similar groupings were observed with PFGE. The majority of swine-derived isolates with the same ST were found to have identical or highly similar PFGE patterns. Conversely, clusters identified by PFGE typically harbored isolates with the same or related STs (among those that were MLST-typed). Large clonal groups based on PFGE with both enzymes typically harbored isolates with the same or closely related STs. MLST typing of three isolates from a PFGE-based cluster of seven swine-derived isolates showed that all three were of the same ST (1157). Similarly, all eight MLST-typed isolates from an especially large PFGE-based cluster of 34 swine isolates were found to be of the same ST (854). As discussed with the turkey isolates, such data suggest the likelihood that the remaining isolates in such clusters were of the same STs.

In the case of *fla*-based clusters, isolates of the same or closely related STs were identified in several small (2-4) clusters of turkey isolates, and similar results were obtained with swine-derived *fla* clusters. However, larger clusters, regardless of the origin (turkeys vs. swine) frequently contained isolates with diverse STs (data not shown).

4.4.5 Association between major clonal groups and antibiotic resistance profiles. In both turkey- and swine-derived isolates, those with identical PFGE profiles (based on *Sma*I and *Kpn*I) and identical STs typically also had antibiotic resistance profiles that were either the same or differed only in regard to one of the antibiotics. For instance, ST-854 swine-derived isolates with the identical PFGE profiles had the same resistance

profile (resistance to tetracycline, streptomycin, and erythromycin), and ST-1150 turkey isolates with identical PFGE profile also had the same resistance (to tetracycline and kanamycin). However, some differences in resistance were noted among isolates of the same ST. Thus, the majority of isolates within a turkey- or swine-associated clonal group (identified by PFGE or *fla*-typing) had the same antibiotic resistance profile.

A turkey-associated clone found in 12 isolates (from four different farms) was present on one farm (T1) for the entire survey period, with the majority having the same resistance profile (TKQ). Two STs (1150 and 1487) from two farms (T2 and T6) were observed among the clonal group, respectively. These STs were closely related; however, differed in antibiotic resistance (TK and TKQ). Two large clonal groups were observed among swine, containing 14 and 18 isolates, respectively. The first group of isolates (n= 14) derived from three farms, and most of them were resistant to tetracycline and erythromycin. Two isolates within this group were of ST-1096 (same resistance profile, TE), but from two different farms (S5, S7). Five isolates of ST-854 were found in the largest swine-associated clonal group (n=18), from three farms (S5, S6, S7) and the majority were resistant to TSE (one other was resistant to TSEK).

With *Kpn*I, fewer large clonal groups were found among turkeys and swine. The maximum number of isolates that one turkey-associated clone could be found was six isolates. There was a lot of variation in the resistance profiles of one clonal group (n= 6). The isolates derived from four different farms, with two STs being detected (1150, 1161) in three isolates from three different farms. Although the STs were closely related (Fig. 4.1) their resistance profiles differed greatly (Q, TK, TSQ). A swine-associated clone was present in eight isolates from two farms and different herds on farm S7, with TE

being the most prevalent profile. Two isolates with ST-1836 from farm S7 (at different time points) had the same resistance profile (TE).

Similar associations were identified by a *fla*-based analysis also. Two turkey isolates of the same clone were from farm T2 (at different time points) and multi-drug resistant (TSEKQ); however, two different STs (1101, 1163) were detected, but determined by MLST to be related STs (Fig. 4.1). Another clonal group of five isolates were all multi-drug resistant (TSEKQ), deriving from farm T1 and one isolate found to be of ST-1161. Two large swine-associated clonal groups (39 and 54 isolates) were each found to have two isolates with the same antibiotic resistance pattern (TSEK and TE), but different STs (829, 1096 and 1113, 1246), respectively. Interestingly, the swine-associated STs detected in either group were not closely related STs, but had the same clone.

4.5 DISCUSSION

Regardless of subtyping method, detectable clusters of strain types were host-specific. Turkeys had a more genetically diverse population, as compared to swine. Such findings suggest that *C. coli* was more host-adapted in regards to swine than to turkeys. The organism was found to frequently colonize turkeys (Chapters 2 and 3); however, appeared not to have fully adapted to the host. Of the 29 STs from swine, 28 were clustered in swine-associated groups, and only one (ST-1164) was identified in swine but grouped with turkey-associated STs. All swine-associated STs derived from ST-828, a ST also detected in a swine isolate. These findings suggest that all swine-associated STs originated from a common ancestor, of swine origin.

Turkey-associated STs were all clustered in three groups, with two of the groups deriving from swine-associated STs (828 and 1055). STs in these two groups may correspond to turkey-associated strains which evolved from swine-associated ancestors and subsequently adapted to turkeys. The third cluster (cluster II) of turkey-derived STs lacked noticeable relatedness to swine-derived STs; thus, no readily identifiable origin seems to exist for this cluster, and cluster II strains are especially host-adapted (to turkeys). There is little genetic similarity between turkey-derived STs in cluster I and those in cluster II, suggesting that these campylobacters colonizing turkeys have derived from different ancestral strains and thus harbor significant divergence.

Analysis of host strains with a single enzyme determined strain-specific trends among the surveyed farms, indicating persistence of strains between flocks (or herds) and dissemination of strains among farms involved in the study. Although many different strain types were detected among turkeys and swine, PFGE using *Sma*I or *Kpn*I was unable to detect clusters containing both turkey- and swine-derived isolates. Conversely, *fla*-typing based clusters containing isolates from both animal sources were identified. A larger number of types were identified with PFGE using *Kpn*I and *fla*, as compared to PFGE using *Sma*I. Such findings indicate that sites recognized by *Kpn*I or *fla* are more variable. Typically, larger clusters of indistinguishable types contained isolates from different farms, including those from different time points. Detection of the same organism at different time points on the same farm indicates that the strain persisted throughout production. Other noticeably large clusters contained the same organisms from different farms, and those from different survey periods of the same farm. These particular strains appear to have disseminated among the surveyed flocks (or herds).

In this study, many more isolates were subtyped with PFGE than MLST.

There was good correlation between MLST and PFGE subtyping data, with isolates having identical PFGE types also having the same or closely related STs, and vice versa. Thus, the identification of a specific ST in MLST-typed isolates of a clonal group with identical PFGE types suggest the likely presence of the same or closely related STs among the remaining isolates of the clonal group. Thus, ST-854, found in six of the 62 MLST-typed swine isolates, may be found in many other isolates, since a large cluster of 34 isolates with the same or closely related PFGE type included several of ST-854. Similarly, cluster II STs, found in 18 of the 37 turkey isolates typed by MLST, would be expected to be found in many other turkey-derived isolates. For identified clusters consisting of isolates that derived from the same farm but differed in antibiotic resistance patterns, it is highly likely that these strains have the same ST, despite the fact that neither or only one strain was typed. The difference in antibiotic resistance might suggest the acquisition or loss of resistance markers by some of the strains. MLST-based subtyping of additional isolates will be needed to substantiate the prevalence of the different STs among turkey- and swine-derived isolates.

Prevalent clones were detected among each host population, and these clonal groups appeared to also be host-specific. A majority of turkey- and swine-associated clonal groups derived from different farms (at the same or various time points), but there were instances in which these clones were detected between survey periods of the same farm. Strain types that were found among different farms and between survey periods suggest the presence of stable clones. These turkey- and swine-derived clonal groups were frequently associated with certain antibiotic resistance patterns in this study. Most

turkey-associated clones were resistant to the entire panel of antibiotics (TSEKQ), whereas the resistance profile TSE was frequently observed among swine-associated clones, suggesting that these stable clones have acquired resistance to multiple drugs over time. Since many of the antibiotics were not used during the course of the study it seems that clones with pre-existing antibiotic resistance profiles persisted throughout production; alternatively, the bacteria may have acquired resistance due to exposure to structurally-related agents, or via the acquisition (via horizontal transfer, e.g. transformation) of elements conferring multiple resistance attributes (e.g. a plasmid or transposon).

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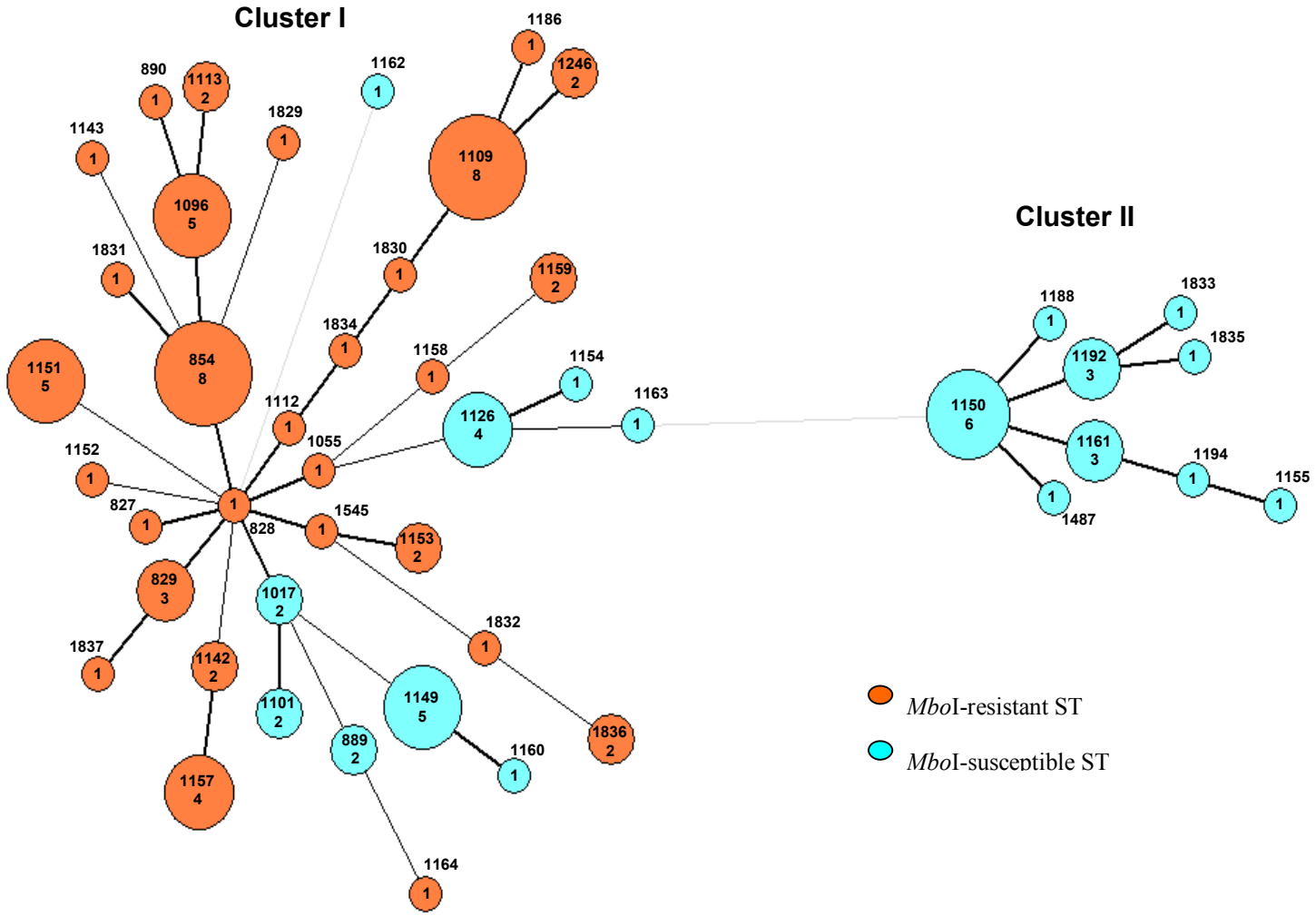

Figure 4.1 Minimum spanning tree depicting *C. coli* strains from turkeys and swine characterized by MLST

5. Methylation at GATC sites in genomic DNA of *Campylobacter coli* from turkeys and swine

5.1 ABSTRACT

In the process of characterizing *C. coli* from turkeys and swine in eastern North Carolina, we investigated prevalence of methylation at adenine or cytosine of GATC sites in the genome of the organisms. Examination of 187 *C. coli* isolates, including 107 from swine and 80 from turkeys, failed to identify any with DNA resistant to digestion by *Sau*3AI, suggesting the absence of cytosine methylation at GATC sites in the DNA of the organisms. However, a significant fraction (43%) of the 107 swine isolates had genomic DNA that was resistant to digestion by the restriction enzyme *Mbo*I, suggesting methylation of adenines at GATC sites. None of the 80 tested *C. coli* isolates from turkeys harbored such methylation. Four swine-associated multilocus sequence typing-based sequence types (STs) were detected in several of the strains with *Mbo*I-resistant DNA. The data suggest a pronounced host-associated difference in prevalence of DNA modification systems specific for adenines at GATC sites in the genome of *C. coli* from turkeys and swine, with methylation being highly prevalent in the former but not detectable in the latter.

5.2 INTRODUCTION

Campylobacter coli commonly infects swine, but has also been isolated from turkeys in eastern North Carolina and other regions of the United States (Logue et al, 2003; Smith et al, 2004; Lee et al, 2005; Wesley et al, 2005; Luangtongkum et al , 2006). A recent analysis of *C. coli* strains from different meat animals by multilocus sequence typing (MLST) revealed presence of several sequence types (STs) that were associated

with specific hosts (Miller et al, 2006). Characterization of antimicrobial susceptibility profiles in *C. coli* derived from different animals has shown noticeable differences in prevalence of resistance to several antibiotics (Saenz et al, 2000). We have observed similarly pronounced differences in antibiotic susceptibility profiles of *C. coli* derived from turkeys versus those from swine in eastern North Carolina (S. Wright, R. Siletzky, and S. Kathariou, unpublished). In addition, we have found that *C. coli* from turkeys are more prone to acquire resistance to erythromycin via natural transformation than their counterparts from swine (Kim et al, 2006). Such findings suggest that *C. coli* from different meat animals harbor genetic attributes that confer to them distinct phenotypes and host-associated adaptations. However, with the exception of antibiotic susceptibility profiles and the afore-mentioned ability to acquire erythromycin resistance by natural transformation, such host-associated phenotypes remain largely uncharacterized. To date only one *C. coli* strain (RM2228, a multi-drug resistant strain derived from chicken) has had its genome completely sequenced (http://www.plosbiology.org) (Fouts et al, 2005), and genome-wide comparisons of *C. co*li from different animal hosts have not been described.

DNA modification at specific sites is a common feature of bacterial genomes, and is frequently associated with restriction-modification systems (Low et al, 2001). A type of DNA modification that has been extensively identified in various bacterial genomes involves methylation of either adenines or cytosines at GATC sites, rendering the genomic DNA of the organism resistant to enzymes such as *Mbo*I and *Sau*3AI, respectively (Barbeyron et al, 1984; Edmonds et al, 1992; Bolstad and Jensen, 1993). Cytosine methylation at GATC sites is frequently encountered among bacterial

pathogens, and in several species it has been found to be characteristic of specific clonal groups (Zheng and Kathariou, 1997; Yildirim et al, 2004). On the other hand, adenine methylation systems at GATC sites are encountered not only as part of type II restriction-modification systems, but also as solitary DNA methylation systems (e.g. the DNA adenine methylation system of *Escherichia coli*, *dam*) of key importance to DNA mismatch repair mechanisms and a variety of regulatory processes (Lobner-Oleson et al, 2005).

Surprisingly limited information is available on the presence of either adenine or cytosine modification at GATC sites in *Campylobacter* species. A survey of 12 *Campylobacter* strains identified three (one each of *C. jejuni*, *C. coli*, and *C. upsaliensis*) that harbored methylated adenines at GATC sites, and none of the isolates harbored methylated cytosines at these sites (Edmonds et al, 1992). However, only two strains of *C. coli* were included in the survey, and their sources were not identified.

To further characterize *C. coli* from meat animals, particularly from turkeys and swine where *C. coli* appears to be especially prevalent, it is desirable to identify possible host-associated attributes that differentiate organisms from these different hosts. DNA methylation and restriction-modification systems would be of special interest due to their potential to impact gene transfer among organisms of the same species. The objective of this study was to investigate the prevalence of adenine or cytosine methylation at GATC sites in the genomic DNA of *C. coli* from turkeys and swine.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial strains and culture conditions. The swine-derived *Campylobacter coli* strains investigated in this study are listed in Table 5.1. These organisms were derived

from different swine farms and were chosen to represent diverse strains (based on PFGE and MLST) and antibiotic resistant profiles, and were isolated between 2003 and 2005. In addition, we investigated 80 turkey-derived *C. coli* isolates from our laboratory's *Campylobacter* strain collection. The turkey isolates were derived from 15 different flocks representing 21 farms between 2003 and 2005, and included organisms with diverse genomic fingerprints based on PFGE and MLST.

Both turkey-derived and swine-derived isolates were isolated as described earlier (Smith et al, 2004) from direct plating of fecal samples. Bacteria were routinely grown on blood agar medium (Remel, Lenexa, KS), as described previously (Smith et al, 2004). Cultures were incubated under microaerophilic conditions at 42˚C for 48 hours. A single colony was obtained as a representative sample of each culture, and each culture was stored in brain heart infusion media (Becton, Dickson, and Company, Sparks, Maryland) with 20% glycerol, at -80ºC.

5.3.2 Bacterial subtyping by multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). MLST amplification and sequencing, as well as assignment of allele numbers and STs, were performed as described previously (Miller et al, 2006). Primer sets used for amplification included: aspAF1/aspAR1, atpAF/atpAR, glnAF/glnAR, gltAF/gltAR, glyAF/glyAR, pgmF1/pgmR1, and tktF1/tktR1 (Miller et al, 2005). The *C. jejuni*/*C. coli* MLST database (http://pubmlst.org/campylobacter/) (Jolley et al, 2004) was used to reference the allelic sequences obtained in the current study. A minimum spanning tree of STs was constructed by Bionumerics (version 3.5; Applied Maths).

PFGE-typing of bacterial isolates was performed as previously described by Ribot et al (2001). Agarose plugs were prepared, lysed, washed, and underwent a restriction digestion with *Sma*I, as well as a secondary enzyme, *Kpn*I. Electrophoretic conditions used were those described previously (Ribot et al, 2001). A dendogram UPGMA analysis of the PFGE profiles was created with Bionumerics (version 3.5; Applied Maths), with 2% optimization and band position tolerance.

5.3.3 Antibiotic susceptibility determinations. Susceptibility to six antibiotics, with varying concentrations, was determined as described before (Lee et al, 2005), employing the NCCLS (currently CSLI) protocol and using *C. jejuni* ATCC 33560 (purchased from ATCC) as the quality control strain. Growth of the bacteria was monitored on Mueller-Hinton Agar plates containing the respective antibiotics, following incubation at 42˚C for 48 hours.

5.3.4 Chromosomal DNA extractions and DNA restrictions. Chromosomal DNA was extracted as described previously (Smith et al, 2004). Enzymatic digestions with *Sau*3AI and *Mbo*I (New England Biolabs, Waverly, Mass.) were employed to determine methylation at cytosine or adenine, respectively, at GATC sites. Selected isolates (n=18) were digested with *Dpn*I (New England Biolabs), which cuts DNA at GATC sites only when the adenine is methylated. Restriction enzyme digestions were done according to the suggestions of the vendor (New England Biolabs).

5.4 RESULTS

5.4.1 Prevalence of GATC methylation in *C. coli* from turkeys and swine. Genomic DNAs from a total of 187 *C. coli* isolates, including 107 from swine and 80 from turkeys, were tested for GATC methylation (Table 5.1). Resistance to *Sau*3AI restriction

indicates methylation at the cytosine residue of GATC sites, whereas methylation at the adenine residue of these sites is expected to result in resistance to *Mbo*I digestion. DNA from all 80 turkey isolates was readily digestible with both *Sau3*AI and *Mbo*I (data not shown), suggesting absence of methylation at either the adenine or the cytosine at GATC sites.

In contrast, of the 107 swine-derived isolates, 46 (43%) yielded DNA that was resistant to digestion by *Mbo*I (Table 5.1). The DNA of these organisms could be readily digested by *Sau*3AI, suggesting that the resistance to digestion by *Mbo*I was due to methylation of adenine at the GATC sites, and not to impurities in the DNA preparation that might interfere with the restrictions. A subset of the *Mbo*I-resistant isolates was also digested with *Dpn*I, and all yielded DNA that was digested by *Dpn*I (Table 5.1). The swine isolates with *Mbo*I-resistant DNA were derived from several different farms, and at various time points between October 2003 and November 2004 (Table 5.1).

5.4.2 Genetic diversity among *C. coli* from swine with adenine methylation at GATC sites. PFGE analysis of the 107 swine-derived *C. coli* isolates revealed one PFGE-based cluster (A) (profile similarity exceeding 77%) that contained only isolates with *Mbo*I-resistant DNA (Table 5.2 and Fig. 5.1). On the other hand, another PFGE-based cluster (B, profile similarity exceeding 79%) of isolates with closely related genomic fingerprints included organisms with *Mbo*I-resistant as well as *Mbo*I-susceptible DNA (Table 5.2 and Fig. 5.2). A third PFGE-based cluster (C, 71% similarity) contained six *Mbo*I-resistant and one *Mbo*I-susceptible isolates (Table 5.2).

MLST-based sequence types (STs) were determined for 62 of the swine-derived *C. coli* isolates, including 24 and 38 isolates with DNA that was *Mbo*I-resistant and

*Mbo*I-susceptible, respectively. Twenty-nine STs were observed among these swine isolates, with 12 of these being encountered in one or more isolates with *Mbo*I-resistant DNA. *Mbo*I-resistant isolates tended to be closely related, being placed in common branches in a minimum spanning tree depicting putative relationships among the STs (Fig. 5.3).

Four STs (854, 1096, 1109, 1246) were found in multiple strains with *Mbo*I-resistant DNA. All five isolates of ST-1096 and seven of the eight isolates of ST-1109 were *Mbo*I-resistant. In contrast, only two of the eight isolates of ST-854 were resistant to *Mbo*I digestion (Table 5.3). One of the two *Mbo*I-resistant isolates of ST-854 (6548) was found to have a PFGE profile indistinguishable from that of a *Mbo*I-susceptible isolate of the same ST, and were members of PFGE cluster B, described above, a large cluster that included both *Mbo*I-resistant and *Mbo*I-susceptible organisms (Fig. 5.2 above). Several STs encountered among isolates with *Mbo*I-resistant DNA (1096, 1109, 1112, 1143, 1186, 1246, 1830) were closely related with STs such as 828, 1113, 1152, 1153, 1159, 1545, and 1834, that were only identified in isolates with *Mbo*I-susceptible DNA (Table 5.2).

5.4.3 Prevalence of resistance profiles in *Mbo*I-resistant vs. sensitive swine strains. Nine antibiotic resistance profiles were observed among the 107 swine isolates that were tested for GATC methylation (Table 5.4). TSE (tetracycline, streptomycin, erythromycin) and TE (tetracycline, erythromycin) were the resistance profiles most frequently observed among both *Mbo*I-resistant and *Mbo*I-susceptible isolates. The profile T (tetracycline resistance only) was seen in 4 (7%) of the *Mbo*I-susceptible strains, but was not detected in those with resistance.

5.5 DISCUSSION

Host-specific differences were observed among turkey and swine isolates that were tested for methylation at GATC sites. Methylation of adenines at GATC sites was detected in 43% of swine-derived *C. coli* isolates. However, none of the turkey isolates were methylated at the adenine at GATC sites. Such findings suggest that methylation at GATC sites is host-specific, meaning certain hosts may carry or acquire methylase genes while others do not. The majority of swine isolates with *Mbo*I-resistant DNA represented different farms, and were isolated at different time points. Isolates with *Mbo*I-resistant DNA were found between different herds (or survey periods) on farms S1 and S4, suggesting persistence of the strains on these farms, or widespread occurrence of the strains in swine farms.

Twelve STs were identified as having one or more strains with *Mbo*I-resistant DNA, with four STs (854, 1096, 1109, 1246) being detected in multiple strains. The clonal ST groups 1096 and 1109 had all five isolates and seven out of eight isolates that were *Mbo*I-resistant, respectively. Several STs among isolates with *Mbo*I-resistant DNA were closely related to STs identified in isolates with *Mbo*I-susceptible DNA. The lack of methylation of adenines at GATC sites in such strains proposes that either the methylase genes were lost at some time (potentially due to a mutation or deletion) or not yet acquired by the sensitive strains. It is quite possible that the resistant strains acquired genes through horizontal transfer or natural transformation, an advantage not seen in the ST-854 sensitive strains. Strains of the same organism may harbor different methylases that methylate adenine; therefore, host-associated (turkeys vs. swine) factors may play a role in these restriction-modification systems. Methylase genes may be accompanied by

restriction enzymes, but not at all times. Swine may have the restriction enzyme to cleave foreign DNA, which may lead to methylated DNA among host strains and the acquisition of *Mbo*I resistance. However, the accompanying enzyme may not be present in turkeys, which improves the chances of foreign DNA being acquired; thus, these strains show susceptibility to *Mbo*I.

Further studies are needed to characterize methylase genes of *Campylobacter* strains from different hosts, which would help to explain host-specific factors observed in the current study (e.g. genes were present in swine and not in turkeys). The presence of methylase genes may be an inherent or acquired attribute depending upon the host; however, the mechanisms involved are unknown. In a growing collection of markers differentiating *C. coli* in turkeys and swine, the acquisition of methylase genes, or lack thereof, may influence certain host-associated issues (e.g. antibiotic resistance) in which different hosts acquire resistance to certain antibiotics, with or without exposure. If accompanied by restriction enzymes, these methylase genes may abate the ability of the surveyed swine herds to acquire fluoroquinolone (FQ) resistance, when in close proximity to turkeys (to which FQ treatment is administered) and vice versa in regards to erythromycin resistance.

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Table 5.1 Swine-derived *C. coli* strains characterized by methylation at GATC sites

ID	Received	Farm	AB^R Profile	MLST	Methylation	*Sau*3A	*Mbo*I	*Dpn*I
5973	10/31/2003	McCullen	TK	1142	Sau3A^s, MboI^s	+	+	
5974	10/31/2003	McCullen	TSE	1151	Sau3A^s, MboI^s	+	+	
5976	10/31/2003	McCullen	TSEK	1151	Sau3A^s, MboI^s	+	+	
5977	10/31/2003	McCullen	T	1152	Sau3A^s, MboI^s	+	+	
5978	10/31/2003	McCullen	TSE	827	Sau3A^s, MboI^s	+	+	
5979	10/31/2003	McCullen	TSK	1153	Sau3A^s, MboI^s	+	+	
5980	10/31/2003	McCullen	TS	1153	Sau3A^s, MboI^s	+	+	
5981 h-	10/31/2003	McCullen	TSE	1112	Sau3A^r, MboI^r	+	+	
5983	10/31/2003	McCullen	TE	1096	Sau3A^s, MboI^r	+	-	
5984	10/31/2003	McCullen	TSE	1143	Sau3A^s, MboI^r	+	-	+
5997	11/14/2003	Grantham	TSEK	1246	Sau3A^s, MboI^r	+	-	+
6008	11/14/2003	Grantham	TSEK	1246	Sau3A^s, MboI^r	+	-	
6022	11/14/2003	McCullen	TEK	1142	Sau3A^s, MboI^s	+	+	
6023	11/14/2003	McCullen	TEK	1157	Sau3A^s, MboI^s	+	+	
6029	11/14/2003	McCullen	TSE	1151	Sau3A^s, MboI^s	+	+	
6045 white	12/1/2003	Grantham	TSEK	1158	Sau3A^s, MboI^s	+	+	
6046	12/1/2003	Grantham	TSEK	1113	Sau3A^s, MboI^s	+	+	
6051	12/1/2003	Grantham	TS	1159	Sau3A^s, MboI^s	+	+	
6083	12/5/2003	McCullen	TSE	1109	Sau3A^s, MboI^r	+	-	+
6084	12/5/2003	McCullen	TE	829	Sau3A^s, MboI^s	+	+	
6087	12/5/2003	McCullen	TEK	828	Sau3A^s, MboI^s	+	+	
6093	12/5/2003	McCullen	TEK	1157	Sau3A^s, MboI^s	+	+	
6094	12/5/2003	McCullen	TE	1157	Sau3A^s, MboI^s	+	+	
6114	12/15/2003	Grantham	TS	1159	Sau3A^s, MboI^s	+	+	
6115	12/15/2003	Grantham	TSEK	1113	Sau3A^s, MboI^s	+	+	
6123	12/15/2003	Grantham	TEK	1164	Sau3A^s, MboI^s	+	+	
6153	2/5/2004	Matthews	TE	ND	Sau3A^s, MboI^r	+	-	
6176	2/19/2004	Matthews	TSE	ND	Sau3A^s, MboI^r	+	-	
6225	3/11/2004	Matthews	TSK	ND	Sau3A^s, MboI^s	+	+	
6228	3/11/2004	Matthews	TSEK	ND	Sau3A^s, MboI^r	+	-	
6230	3/11/2004	Matthews	TSK	ND	Sau3A^s, MboI^r	+	-	
6263	3/19/2004	Warren	TSE	854	Sau3A^s, MboI^s	+	+	
6267	3/19/2004	Warren	TSEK	ND	Sau3A^s, MboI^s	+	+	
6269	3/19/2004	Warren	TE	1055	Sau3A^s, MboI^r	+	-	+

Table 5.1 continued

ID	Received	Farm	AB^R Profile	MLST	Methylation	*Sau*3A	*Mbo*I	*Dpn*I
6302	3/25/2004	Butler	TSE	1109	Sau3A^s, MboI^r	+	-	+
6307	3/25/2004	Butler	T	ND	Sau3A^s, MboI^s	+	+	
6324	3/26/2004	Marshall	TE	ND	Sau3A^s, MboI^r	+	-	
6325	3/26/2004	Marshall	TSE	ND	Sau3A^s, MboI^r	+	-	
6335	3/26/2004	Marshall	TSE	ND	Sau3A^s, MboI^r	+	-	
6354	3/31/2004	Matthews	TSE	ND	Sau3A^s, MboI^r	+	-	
6357	3/31/2004	Matthews	TSE	ND	Sau3A^s, MboI^r	+	-	
6358	3/31/2004	Matthews	TS	ND	Sau3A^s, MboI^r	+	-	
6359	3/31/2004	Matthews	TSK	ND	Sau3A^s, MboI^s	+	+	
6362	3/31/2004	Matthews	T	ND	Sau3A^s, MboI^s	+	+	
6461	4/8/2004	Warren	TSE	854	Sau3A^s, MboI^r	+	-	+
6464	4/8/2004	Warren	TSE	1829	Sau3A^s, MboI^r	+	-	+
6465	4/8/2004	Warren	TE	ND	Sau3A^s, MboI^r	+	-	
6468	4/8/2004	Warren	TSE	1109	Sau3A^s, MboI^r	+	-	
6493	4/9/2004	Butler	TE	1109	Sau3A^s, MboI^r	+	-	+
6495	4/9/2004	Butler	T	1109	Sau3A^s, MboI^s	+	+	
6513	4/15/2004	Marshall	TEK	ND	Sau3A^s, MboI^r	+	-	
6517	4/15/2004	Marshall	TSK	ND	Sau3A^s, MboI^s	+	+	
6519	4/15/2004	Marshall	TK	ND	Sau3A^s, MboI^s	+	+	
6540	4/22/2004	Warren	TSEK	1186	Sau3A^s, MboI^r	+	-	+
6542	4/22/2004	Warren	TSK	854	Sau3A^s, MboI^s	+	+	
6544	4/22/2004	Warren	TSEK	1831	Sau3A^s, MboI^r	+	-	+
6546	4/22/2004	Warren	TSEK	1109	Sau3A^s, MboI^r	+	-	+
6548	4/22/2004	Warren	TSEK	854	Sau3A^s, MboI^r	+	-	+
6550	4/22/2004	Warren	TSEK	1096	Sau3A^s, MboI^r	+	-	+
6587	4/29/2004	Marshall	TSE	ND	Sau3A^s, MboI^r	+	-	
6593	4/29/2004	Marshall	TSEK	ND	Sau3A^s, MboI^s	+	+	
6594	4/29/2004	Marshall	TEK	ND	Sau3A^s, MboI^s	+	+	
6595	4/29/2004	Marshall	TSEK	ND	Sau3A^s, MboI^s	+	+	
6616	5/4/2004	Butler	TE	829	Sau3A^s, MboI^s	+	+	
6618	5/4/2004	Butler	TSE	854	Sau3A^s, MboI^s	+	+	
6620	5/4/2004	Butler	TE	1096	Sau3A^s, MboI^r	+	-	
6622	5/4/2004	Butler	TSE	1832	Sau3A^s, MboI^s	+	+	
6625	5/4/2004	Butler	TSE	1151	Sau3A^s, MboI^s	+	+	

Table 5.1 continued

ID	Received	Farm	AB^R Profile	MLST	Methylation	*Sau*3A	*Mbo*I	*Dpn*I
6645	5/6/2004	Warren	TSK	854	Sau3A^s, MboI^s	+	+	
6649	5/6/2004	Warren	TK	1096	Sau3A^s, MboI^r	+	-	+
7025	6/23/2004	McCullen	TS	ND	Sau3A^s, MboI^s	+	+	
7026	6/23/2004	McCullen	TE	ND	Sau3A^s, MboI^s	+	+	
7033	6/23/2004	McCullen	TE	ND	Sau3A^s, MboI^r	+	-	
7034	6/23/2004	McCullen	TSE	ND	Sau3A^s, MboI^r	+	-	
7176	7/6/2004	Marshall	TE	ND	Sau3A^s, MboI^r	+	-	
7181	7/6/2004	Marshall	TE	ND	Sau3A^s, MboI^s	+	+	
7334	7/13/2004	McCullen	TSE	ND	Sau3A^s, MboI^s	+	+	
7341	7/13/2004	McCullen	TS	ND	Sau3A^s, MboI^s	+	+	
7348	7/13/2004	McCullen	TE	ND	Sau3A^s, MboI^r	+	-	
7354	7/13/2004	McCullen	TSE	ND	Sau3A^s, MboI^s	+	+	
7362	7/13/2004	McCullen	TE	ND	Sau3A^s, MboI^s	+	+	
7370	7/13/2004	McCullen	SE	ND	Sau3A^s, MboI^r	+	-	
7831	8/5/2004	Marshall	TE	ND	Sau3A^s, MboI^s	+	+	
7834	8/5/2004	Marshall	TEK	ND	Sau3A^s, MboI^s	+	+	
7840	8/5/2004	Marshall	TSE	ND	Sau3A^s, MboI^s	+	+	
7941	8/11/2004	McCullen	TE	ND	Sau3A^s, MboI^s	+	+	
7950	8/11/2004	McCullen	TE	ND	Sau3A^s, MboI^s	+	+	
7955	8/11/2004	McCullen	TSE	ND	Sau3A^s, MboI^r	+	-	
7974	8/11/2004	McCullen	TSE	ND	Sau3A^s, MboI^r	+	-	
7977	8/11/2004	McCullen	TE	ND	Sau3A^s, MboI^s	+	+	
8108	8/20/2004	Marshall	TSE	ND	Sau3A^s, MboI^r	+	-	
8112	8/20/2004	Marshall	TSE	ND	Sau3A^s, MboI^r	+	-	
8425	9/22/2004	West	TSE	1834	Sau3A^s, MboI^s	+	+	
8445	9/22/2004	West	TE	890	Sau3A^s, MboI^r	+	-	+
8689	10/6/2004	West	TEK	1109	Sau3A^s, MboI^r	+	-	+
8690	10/6/2004	West	TSE	1836	Sau3A^s, MboI^s	+	+	
8697	10/6/2004	West	TE	1837	Sau3A^s, MboI^s	+	+	
8710	10/6/2004	West	TSE	1096	Sau3A^s, MboI^r	+	-	+
8915	10/27/2004	West	TSE	1545	Sau3A^s, MboI^s	+	+	
8923	10/27/2004	West	TE	829	Sau3A^s, MboI^s	+	+	
8927	10/27/2004	West	TEK	1157	Sau3A^s, MboI^s	+	+	
8943	10/27/2004	West	TSE	854	Sau3A^s, MboI^s	+	+	

Table 5.1 continued

ID	Received	Farm	AB^R Profile	MLST	Methylation	*Sau*3A	*Mbo*I	*Dpn*I
9085	11/17/2004	West	TSE	1109	Sau3A^s, MboI^r	+	-	+
9091	11/17/2044	West	TE	1836	Sau3A^s, MboI^s	+	+	
9093	11/17/2004	West	TSE	854	Sau3A^s, MboI^s	+	+	
9095	11/17/2004	West	TSE	ND	Sau3A^s, MboI^r	+	-	
9104	11/17/2004	West	TSE	1151	Sau3A^s, MboI^s	+	+	

Table 5.2 PFGE clusters of swine-derived *C. coli* strains with *Mbo*I-resistant and *Mbo*I-susceptible DNA

Cluster	No. of Strains (# Resistant)	MLST (and other comments)
A	10 (10)	1096 (4 R), 6 R strains not MLST-typed
B	11 (2)	854 (2 R[1], 6 S[1]), 3 S strains not MLST-typed
C	19 (14)	1109 (5 R, 1 S); 890, 1831, 1186 (all had 1 R); 1246 (2 R); 1113 (2 S); 4 R and 2 S strains not MLST-typed
D	12 (3)	827, 1837 (both had 1 S); 829 (3 S); 3 R and 4 S strains not MLST-typed
E	3 (2)	1143 (1 R), 828 (1 S), 1 R strain not MLST-typed
F	7 (2)	1112 (1 R); 1834 (1 S), 1 R and 4 S strains not MLST-typed
G	13 (3)	1829 (1 R), 2 R and 3 S strains not MLST-typed
H	10 (6)	1109 (2 R), 1159 (2 S), 1545 (1 S), 4 R and 1 S strain not MLST-typed
I	4 (1)	1096 (1 R), 1152 (1 S), 1153 (2 S)
J	1(1)	1055 (1 R)

[1]R or S: resistant or susceptible

Table 5.3 STs encountered in *C. coli* strains from swine with *Mbo*I resistance

ST	No. of strains	No. of resistant strains
854	8	2
1096	5	5
1109	8	7
1246	2	2
Others*	7	7

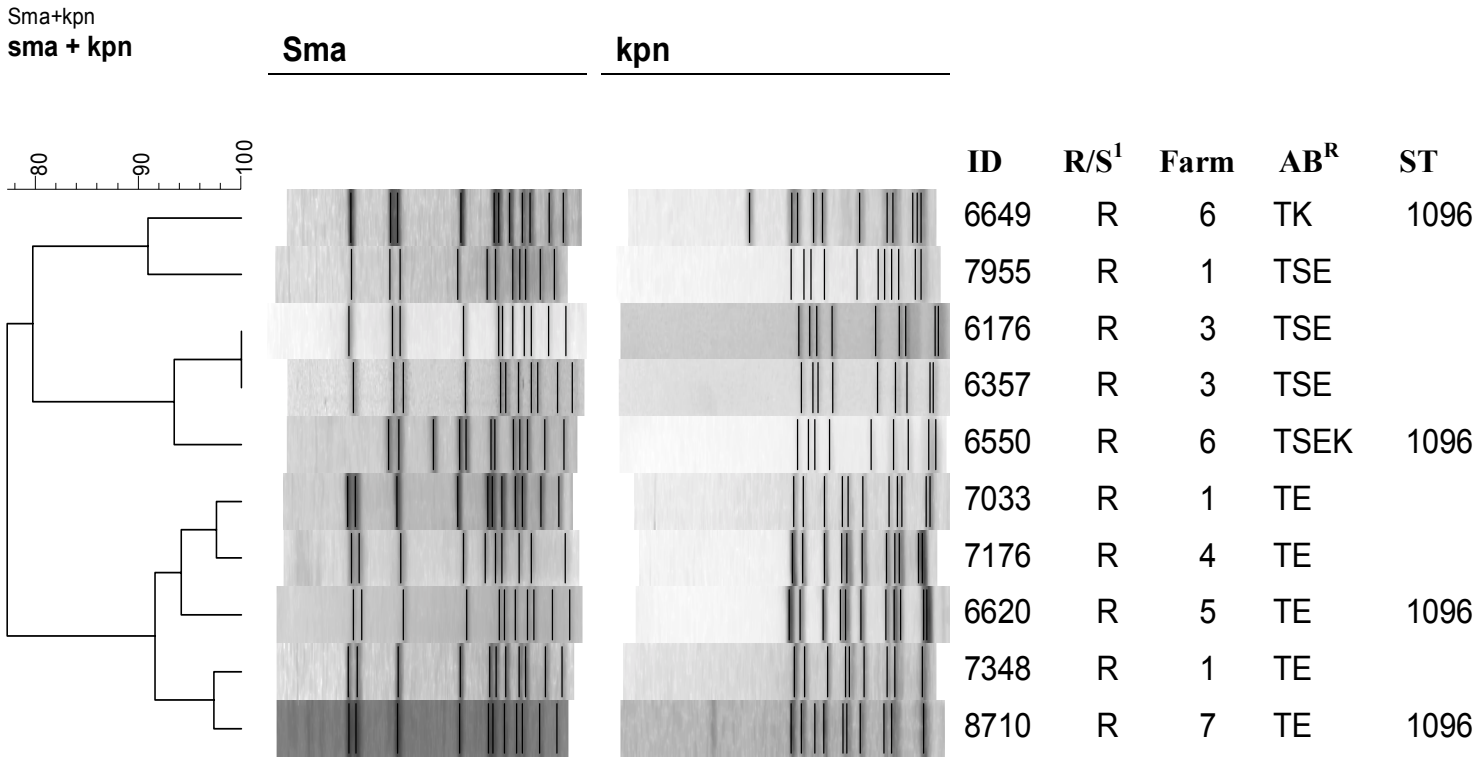
*ST groups with only one strain

Table 5.4 Prevalence of antibiotic resistance profiles in *Mbo*I-resistant vs. *Mbo*I-sensitive *C. coli* strains from swine

Resistance Profile	*Mbo*I-Resistant (n= 46)		*Mbo*I-Sensitive (n= 61)	
T	0	(0)[1]	4	(7)
SE	1	(2)	0	(0)
TE	11	(24)	13	(21)
TK	1	(2)	2	(3)
TS	1	(2)	5	(8)
TEK	2	(4)	8	(13)
TSE	21	(46)	16	(26)
TSK	1	(2)	6	(10)
TSEK	8	(17)	7	(11)

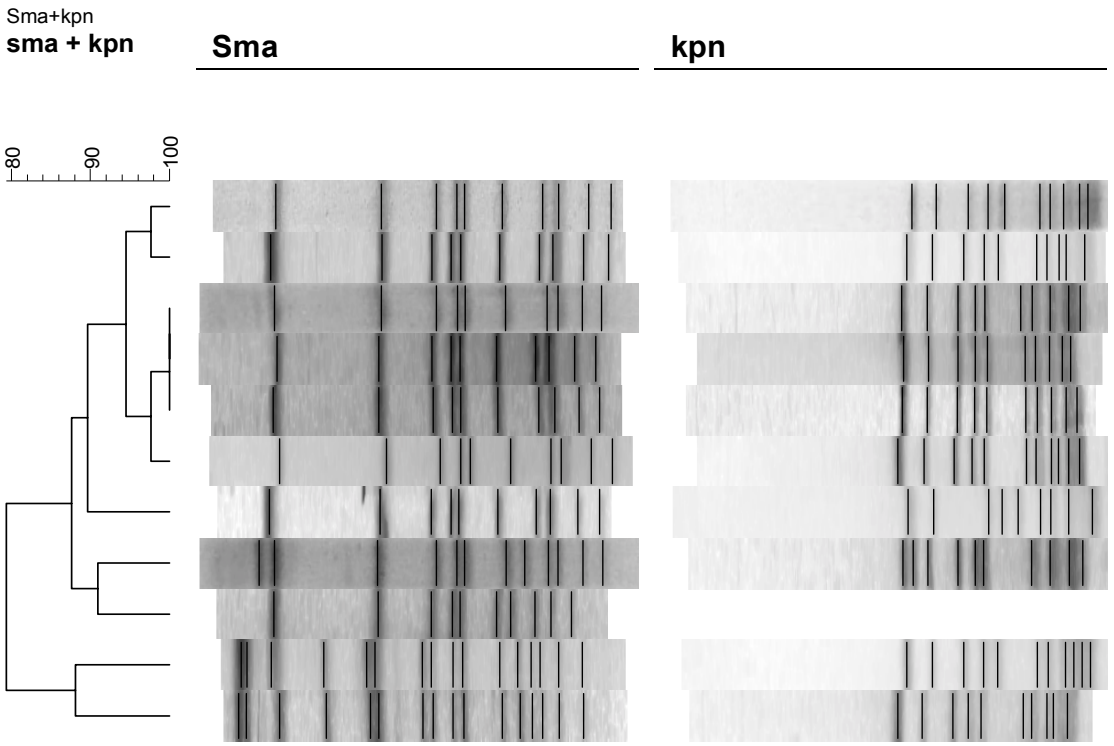
n indicates the number of isolates that were either *Mbo*I-resistant or *Mbo*I-sensitive

[1] Percentages in parentheses



[1]R/S: Resistant or Susceptible to methylation at GATC sites

Figure 5.1 PFGE analysis of *Mbo*I-resistant *C. coli* strains from swine with ST-1096 (Cluster A)



ID	R/S[1]	Farm	AB[R]	ST
6517	S	4	TSK	
6548	R	6	TSEK	854
6263	S	6	TSE	854
7840	S	4	TSE	
9093	S	7	TSE	854
6618	S	5	TSE	854
6461	R	6	TSE	854
6267	S	6	TSEK	
8943*	S	7	TSE	854
6542	S	6	TSK	854
6645	S	6	TSK	854

[1]R/S: Resistant or Susceptible to methylation at GATC sites

[*]No *Kpn*I type, comparison to other isolates based on *Sma*I type only

Figure 5.2 PFGE analysis of *Mbo*I-resistant and *Mbo*I-susceptible *C. coli* strains of ST-854 (Cluster B)

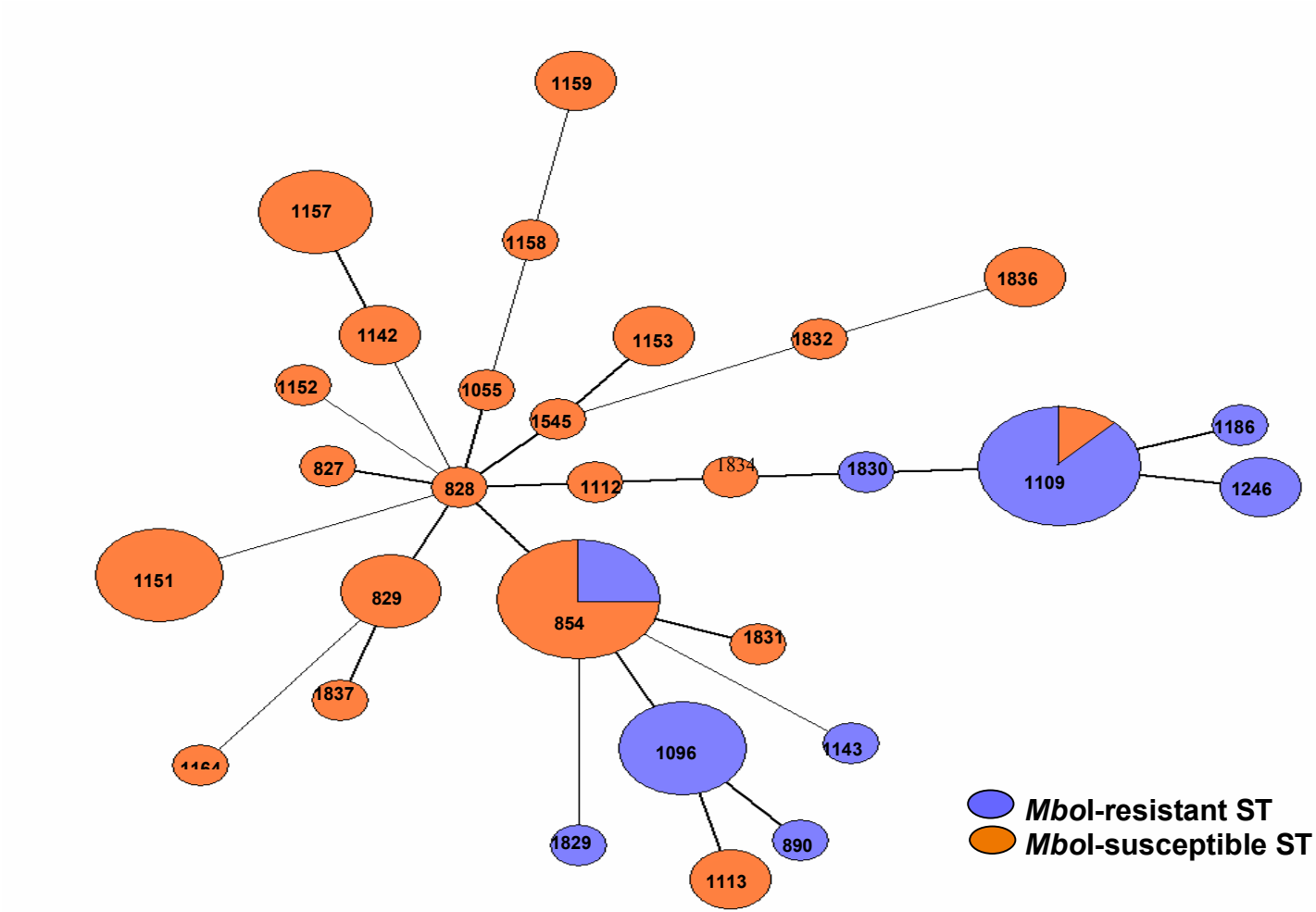


Figure 5.3 Swine-derived *Mbo*I-resistant and -susceptible STs

6. APPENDICES

Appendix 6.1 Turkey-derived *C. coli* strains selected for further characterization by molecular subtyping tools

ID	Received	Farm	Age	Resistance Profile	MLST	Methylation
5961	10/31/2003	1	5.5 wk	MAR	1149	Sau3A^{s}, MboI^{s}
5963	10/31/2003	1	5.5 wk	MAR	1126	Sau3A^{s}, MboI^{s}
5964	10/31/2003	1	5.5 wk	MAR	ND	Sau3A^{s}, MboI^{s}
5965	10/31/2003	1	5.5 wk	MAR	ND	Sau3A^{s}, MboI^{s}
5968	10/31/2003	1	5.5 wk	MAR	ND	Sau3A^{s}, MboI^{s}
5971	10/31/2003	1	5.5 wk	TSKQ	1150	Sau3A^{s}, MboI^{s}
5988	11/14/2003	2	5 1/2 wk	MAR	1101	Sau3A^{s}, MboI^{s}
5991	11/14/2003	2	5 1/2 wk	MAR	1154	Sau3A^{s}, MboI^{s}
5992	11/14/2003	2	5 1/2 wk	TEQ	ND	Sau3A^{s}, MboI^{s}
5993	11/14/2003	2	5 1/2 wk	MAR	ND	Sau3A^{s}, MboI^{s}
5995	11/14/2003	2	5 1/2 wk	MAR	ND	ND
6010	11/14/2003	1	7.5 wk	TSQ	ND	ND
6014	11/14/2003	1	7.5 wk	TKQ	1155	Sau3A^{s}, MboI^{s}
6015	11/14/2003	1	7.5 wk	MAR	ND	ND
6017	11/14/2003	1	7.5 wk	TSKQ	1150	Sau3A^{s}, MboI^{s}
6033	12/1/2003	2	8 wk	TK	1150	Sau3A^{s}, MboI^{s}
6034	12/1/2003	2	8 wk	TQ	ND	ND
6036	12/1/2003	2	8 wk	TK	ND	ND
6037	12/1/2003	2	8 wk	MAR	ND	ND
6039	12/1/2003	2	8 wk	MAR	ND	ND
6043	12/1/2003	2	8 wk	TQ	ND	ND
6067	11/14/2003	1	7.5 wk	TQ	1150	Sau3A^{s}, MboI^{s}
6072	12/5/2003	1	10.5 wk	MAR	1160	Sau3A^{s}, MboI^{s}
6074	12/5/2003	1	10.5 wk	MAR	ND	ND
6076	12/5/2003	1	10.5 wk	MRA	1126	Sau3A^{s}, MboI^{s}
6077	12/5/2003	1	10.5 wk	TSQ	1161	Sau3A^{s}, MboI^{s}
6079	12/5/2003	1	10.5 wk	TSKQ	1162	Sau3A^{s}, MboI^{s}
6098	12/15/2003	2	10 wk	TK	ND	ND
6099	12/15/2003	2	10 wk	TSKQ	ND	ND
6100	12/15/2003	2	10 wk	MAR	1161	Sau3A^{s}, MboI^{s}
6101	12/15/2003	2	10 wk	TSEK	ND	ND
6108	12/15/2003	2	10 wk	MAR	1163	Sau3A^{s}, MboI^{s}
6135	2/5/2004	3	5 wk	TQ	ND	ND
6138	2/5/2004	3	5 wk	TQ	ND	ND
6140	2/5/2004	3	5 wk	MAR	ND	Sau3A^{s}, MboI^{s}
6141	2/5/2004	3	5 wk	MAR	ND	ND
6143	2/5/2004	3	5 wk	TK	ND	ND
6144	2/5/2004	3	5 wk	TQ	ND	ND
6145	2/5/2004	3	5 wk	MAR	ND	ND
6170	2/19/2004	3	7 wk	TQ	ND	Sau3A^{s}, MboI^{s}
6208	3/11/2004	3	10 wk	TQ	ND	ND
6212	3/11/2004	3	10 wk	MAR	ND	Sau3A^{s}, MboI^{s}
6217	3/11/2004	3	10 wk	TQ	ND	ND
6245	3/19/2004	6	5 wk	TQ	ND	ND
6246	3/19/2004	6	5 wk	KQ	1017	Sau3A^{s}, MboI^{s}
6247	3/19/2004	6	5 wk	T	1192	Sau3A^{s}, MboI^{s}

Appendix 6.1 continued

6248	3/19/2004	6	5 wk	Q	ND	ND
6249	3/19/2004	6	5 wk	TSEK	ND	ND
6250	3/19/2004	6	5 wk	T	ND	ND
6252	3/19/2004	6	5 wk	TKQ	1487	Sau3A^{s}, MboI^{s}
6254	3/19/2004	6	5 wk	TSKQ	ND	ND
6256	3/19/2004	6	5 wk	TK	ND	ND
6282	3/25/2004	5	5 wk	MAR	889	Sau3A^{s}, MboI^{s}
6292	3/25/2004	5	5 wk	MAR	ND	ND
6338	3/31/2004	3	13 wk	MAR	ND	ND
6341	3/31/2004	3	13 wk	TEKQ	ND	Sau3A^{s}, MboI^{s}
6342	3/31/2004	3	13 wk	MAR	ND	ND
6343	3/31/2004	3	13 wk	TK	ND	ND
6346	3/31/2004	3	13 wk	TQ	ND	Sau3A^{s}, MboI^{s}
6444	4/8/2004	6	8 wk	KQ	ND	ND
6445	4/8/2004	6	8 wk	T	ND	ND
6449	4/8/2004	6	8 wk	TEKQ	1149	Sau3A^{s}, MboI^{s}
6473	4/9/2004	5	7 wk	TK	1150	Sau3A^{s}, MboI^{s}
6475	4/9/2004	5	7 wk	MAR	ND	Sau3A^{s}, MboI^{s}
6480	4/9/2004	5	7 wk	TEKQ	1149	Sau3A^{s}, MboI^{s}
6529	4/22/2004	6	10 wk	KQ	1017	Sau3A^{s}, MboI^{s}
6530	4/22/2004	6	10 wk	Q	1194	Sau3A^{s}, MboI^{s}
6602	5/4/2004	5	11 wk	TQ	ND	Sau3A^{s}, MboI^{s}
6603	5/4/2004	5	11 wk	MAR	1149	Sau3A^{s}, MboI^{s}
6627	5/6/2004	6	12 wk	TK	1150	Sau3A^{s}, MboI^{s}
6636	5/6/2004	6	12 wk	TEK	1101	Sau3A^{s}, MboI^{s}
6638	5/6/2004	6	12 wk	TEKQ	1149	Sau3A^{s}, MboI^{s}
6639	5/6/2004	6	12 wk	TSEK	ND	ND
6685	5/17/2004	13	5 wk	MAR	1101	Sau3A^{s}, MboI^{s}
6687	5/18/2004	5	13 wk	TQ	ND	ND
6690	5/18/2004	5	13 wk	T	1833	Sau3A^{s}, MboI^{s}
6692	5/18/2004	5	13 wk	TQ	1192	Sau3A^{s}, MboI^{s}
6693	5/18/2004	5	13 wk	T	ND	ND
6695	5/18/2004	5	13 wk	TQ	ND	ND
6697	5/18/2004	5	13 wk	T	ND	ND
6698	5/18/2004	5	13 wk	MAR	889	Sau3A^{s}, MboI^{s}
6780	6/3/2004	9	4 wk	MAR	1126	Sau3A^{s}, MboI^{s}
6890	6/16/2004	11	9 wk	TQ	1161	Sau3A^{s}, MboI^{s}
6916	6/17/2007	12	4 wk	TQ	1150	Sau3A^{s}, MboI^{s}
7015	6/23/2004	1	5 wk	TK	ND	Sau3A^{s}, MboI^{s}
7016 white	6/23/2004	1	5 wk	TKQ	ND	ND
7016 grey	6/23/2004	1	5 wk	MAR	ND	ND
7020	6/23/2004	1	5 wk	MAR	ND	Sau3A^{s}, MboI^{s}
7279	7/12/2004	8	4 wk	SQ	1242	Sau3A^{s}, MboI^{s}
7286	7/13/2004	1	8 wk	MAR	ND	Sau3A^{s}, MboI^{s}
7288	7/13/2004	1	8 wk	TQ	ND	Sau3A^{s}, MboI^{s}
7295	7/13/2004	1	8 wk	MAR	ND	ND
7297	7/13/2004	1	8 wk	TQ	ND	ND
7298	7/13/2004	1	8 wk	TKQ	ND	Sau3A^{s}, MboI^{s}
7301	7/13/2004	1	8 wk	MAR	ND	Sau3A^{s}, MboI^{s}
7302	7/13/2004	1	8 wk	TK	ND	ND
7312	7/13/2004	1	8 wk	MAR	ND	ND
7317	7/13/2004	1	8 wk	TQ	ND	Sau3A^{s}, MboI^{s}
7321	7/13/2004	1	8 wk	TKQ	ND	ND

Appendix 6.1 continued

7325	7/13/2004	1	8 wk	TKQ	ND	Sau3A^{s}, MboI^{s}
7328	7/13/2004	1	8 wk	TSKQ	ND	ND
7725	8/2/2004	10	12 wk	TQ	1150	Sau3A^{s}, MboI^{s}
7816	8/5/2004	4	10 wk	T	ND	Sau3A^{s}, MboI^{s}
7819	8/5/2004	4	10 wk	T	ND	ND
7821	8/5/2004	4	10 wk	MAR	ND	Sau3A^{s}, MboI^{s}
7891	8/11/2004	1	12 wk	TQ	ND	Sau3A^{s}, MboI^{s}
7905	8/11/2004	1	12 wk	AR	ND	Sau3A^{s}, MboI^{s}
7908	8/11/2004	1	12 wk	TSK	ND	ND
7922	8/11/2004	1	12 wk	T	ND	ND
7927	8/11/2004	1	12 wk	TQ	ND	Sau3A^{s}, MboI^{s}
7931	8/11/2004	1	12 wk	TKQ	ND	ND
7933	8/11/2004	1	12 wk	MAR	ND	Sau3A^{s}, MboI^{s}
8023	8/11/2004	1	11 wk	TQ	1192	Sau3A^{s}, MboI^{s}
8103	8/20/2004	4	12 wk	TSEK	ND	Sau3A^{s}, MboI^{s}
8387	9/22/2004	7	5 wk	MAR	1126	Sau3A^{s}, MboI^{s}
8406	9/22/2004	7	5 wk	MAR	ND	ND
8663	10/6/2004	7	7 wk	TK	1835	Sau3A^{s}, MboI^{s}
8870	10/27/2004	7	10 wk	TK	ND	ND
8880	10/27/2004	7	10 wk	TK	ND	ND
8891	10/27/2004	7	10 wk	MAR	1126	Sau3A^{s}, MboI^{s}
8894	10/27/2004	7	10 wk	TK	ND	ND
8901	10/27/2004	7	10 wk	TQ	1192	Sau3A^{s}, MboI^{s}
8906	10/27/2004	7	10 wk	TQ	ND	ND
9034	11/17/2004	7	13 wk	MAR	ND	ND
9036	11/17/2004	7	13 wk	TK	ND	ND
9039	11/17/2004	7	13 wk	Q	ND	ND
9042	11/17/2004	7	13 wk	TK	ND	ND
9043	11/17/2004	7	13 wk	TSKQ	ND	Sau3A^{s}, MboI^{s}
9045	11/17/2004	7	13 wk	Q	1188	Sau3A^{s}, MboI^{s}
9047	11/17/2004	7	13 wk	TK	ND	ND
9061	11/17/2004	7	13 wk	Q	ND	ND
9066	11/17/2004	7	13 wk	TK	ND	ND
9067	11/17/2004	7	13 wk	Q	1161	Sau3A^{s}, MboI^{s}

Appendix 6.2 Swine-derived *C.coli* strains selected for further characterization by molecular subtyping tools

ID	Received	Farm	Resistance Profile	MLST	Methylation
5973	10/31/2003	1	TK	1142	Sau3A^{s}, MboI^{s}
5974	10/31/2003	1	TSE	1151	Sau3A^{s}, MboI^{s}
5976	10/31/2003	1	TSEK	1151	Sau3A^{s}, MboI^{s}
5977	10/31/2003	1	T	1152	Sau3A^{s}, MboI^{s}
5978	10/31/2003	1	TSE	827	ND
5979	10/31/2003	1	TSK	1153	Sau3A^{s}, MboI^{s}
5980	10/31/2003	1	TS	1153	Sau3A^{s}, MboI^{s}
5981 h+	10/31/2003	1	TSE	ND	ND
5981 h-	10/31/2003	1	TSE	1112	Sau3A^{s}, MboI^{s}
5983	10/31/2003	1	TE	1096	Sau3A^{s}, MboI^{r}
5984	10/31/2003	1	TSE	1143	ND
5997	11/14/2003	2	TSEK	1246	ND
5999	11/14/2003	2	TSEK	ND	ND
6001	11/14/2003	2	TE	ND	ND
6003	11/14/2003	2	TE	ND	ND
6005	11/14/2003	2	TEK	ND	ND
6007	11/14/2003	2	TE	ND	ND
6008	11/14/2003	2	TSEK	1246	ND
6021	11/14/2003	1	TSK	ND	ND
6022	11/14/2003	1	TEK	1142	Sau3A^{s}, MboI^{s}
6023	11/14/2003	1	TEK	1157	Sau3A^{s}, MboI^{s}
6025	11/14/2003	1	TEK	ND	ND
6028	11/14/2003	1	TEK	ND	ND
6029	11/14/2003	1	TSE	1151	Sau3A^{s}, MboI^{s}
6045 grey	12/1/2003	2	TEK	ND	ND
6045 white	12/1/2003	2	TSEK	1158	ND
6046	12/1/2003	2	TSEK	1113	Sau3A^{s}, MboI^{s}
6047	12/1/2003	2	TK	ND	ND
6051	12/1/2003	2	TS	1159	Sau3A^{s}, MboI^{s}
6053	12/1/2003	2	TSEK	ND	ND
6055	12/1/2003	2	TS	ND	ND
6083	12/5/2003	1	TSE	1109	Sau3A^{s}, MboI^{r}
6084	12/5/2003	1	TE	829	Sau3A^{s}, MboI^{s}
6085	12/5/2003	1	TEK	ND	ND
6087	12/5/2003	1	TEK	828	ND
6091	12/5/2003	1	TSK	ND	ND
6093	12/5/2003	1	TEK	1157	ND
6094	12/5/2003	1	TE	1157	ND
6113	12/15/2003	2	TSE	ND	ND
6114	12/15/2003	2	TS	1159	Sau3A^{s}, MboI^{s}
6115	12/15/2003	2	TSEK	1113	Sau3A^{s}, MboI^{s}
6121	12/15/2003	2	TEK	ND	ND
6123	12/15/2003	2	TEK	1164	Sau3A^{s}, MboI^{s}
6153	2/5/2004	3	TE	ND	Sau3A^{s}, MboI^{r}
6156	2/5/2004	3	TEK	ND	ND
6159	2/5/2004	3	T	ND	ND
6160	2/5/2004	3	TE	ND	ND
6176	2/19/2004	3	TSE	ND	Sau3A^{s}, MboI^{r}
6177	2/19/2004	3	TSK	ND	ND
6178	2/19/2004	3	TSEK	ND	ND

Appendix 6.2 continued

ID	Received	Farm	Resistance Profile	MLST	Methylation
6183	2/19/2004	3	TSEK	ND	ND
6187	2/19/2004	3	TEK	ND	ND
6225	3/11/2004	3	TSK	ND	Sau3A^{s}, MboI^{s}
6228	3/11/2004	3	TSEK	ND	Sau3A^{s}, MboI^{r}
6230	3/11/2004	3	TSK	ND	Sau3A^{s}, MboI^{r}
6261	3/19/2004	6	TSE	ND	ND
6263	3/19/2004	6	TSE	854	Sau3A^{s}, MboI^{s}
6265	3/19/2004	6	TSE	ND	ND
6267	3/19/2004	6	TSEK	ND	Sau3A^{s}, MboI^{s}
6269	3/19/2004	6	TE	1055	ND
6270	3/19/2004	6	TSE	ND	ND
6298	3/25/2004	5	T	ND	ND
6300	3/25/2004	5	TS	ND	ND
6302	3/25/2004	5	TSE	1109	Sau3A^{s}, MboI^{r}
6304	3/25/2004	5	TSE	ND	ND
6306	3/25/2004	5	TSE	ND	ND
6307	3/25/2004	5	T	ND	Sau3A^{s}, MboI^{s}
6324	3/26/2004	4	TE	ND	Sau3A^{s}, MboI^{r}
6325	3/26/2004	4	TSE	ND	Sau3A^{s}, MboI^{r}
6327	3/26/2004	4	TSE	ND	ND
6329	3/26/2004	4	TS	ND	ND
6331	3/26/2004	4	TEK	ND	ND
6334	3/26/2004	4	TEK	ND	ND
6335	3/26/2004	4	TSE	ND	Sau3A^{s}, MboI^{r}
6354	3/31/2004	3	TSE	ND	Sau3A^{s}, MboI^{r}
6357	3/31/2004	3	TSE	ND	Sau3A^{s}, MboI^{r}
6358	3/31/2004	3	TS	ND	Sau3A^{s}, MboI^{r}
6359	3/31/2004	3	TSK	ND	Sau3A^{s}, MboI^{s}
6362	3/31/2004	3	T	ND	Sau3A^{s}, MboI^{s}
6461	4/8/2004	6	TSE	854	Sau3A^{s}, MboI^{r}
6462	4/8/2004	6	SE	ND	ND
6464	4/8/2004	6	TSE	1829	Sau3A^{s}, MboI^{r}
6465	4/8/2004	6	TE	ND	Sau3A^{s}, MboI^{r}
6468	4/8/2004	6	TSE	1109	Sau3A^{s}, MboI^{r}
6486	4/9/2004	5	TSE	ND	ND
6487	4/9/2004	5	TE	ND	ND
6491	4/9/2004	5	TSK	ND	ND
6493	4/9/2004	5	TE	1109	Sau3A^{s}, MboI^{r}
6494	4/9/2004	5	TE	ND	ND
6495	4/9/2004	5	T	1109	Sau3A^{s}, MboI^{s}
6496	4/9/2004	5	TSE	1830	ND
6513	4/15/2004	4	TEK	ND	Sau3A^{s}, MboI^{r}
6514	4/15/2004	4	TSEK	ND	ND
6517	4/15/2004	4	TSK	ND	Sau3A^{s}, MboI^{s}
6519	4/15/2004	4	TK	ND	Sau3A^{s}, MboI^{s}
6521	4/15/2004	4	TSK	ND	ND
6522 rnd	4/15/2004	4	TEK	ND	ND
6522 spr	4/15/2004	4	TEK	ND	ND
6523	4/15/2004	4	TEK	ND	ND
6539	4/22/2004	6	TSK	ND	ND
6540	4/22/2004	6	TSEK	1186	Sau3A^{s}, MboI^{r}
6542	4/22/2004	6	TSK	854	Sau3A^{s}, MboI^{s}

Appendix 6.2 continued

ID	Received	Farm	Resistance Profile	MLST	Methylation
6544	4/22/2004	6	TSEK	1831	Sau3A^{s}, MboI^{r}
6546	4/22/2004	6	TSEK	1109	Sau3A^{s}, MboI^{r}
6548	4/22/2004	6	TSEK	854	Sau3A^{s}, MboI^{r}
6550	4/22/2004	6	TSEK	1096	Sau3A^{s}, MboI^{r}
6587	4/29/2004	4	TSE	ND	Sau3A^{s}, MboI^{r}
6589	4/29/2004	4	TEK	ND	ND
6590	4/29/2004	4	TS	ND	ND
6593	4/29/2004	4	TSEK	ND	Sau3A^{s}, MboI^{s}
6594	4/29/2004	4	TEK	ND	Sau3A^{s}, MboI^{s}
6595	4/29/2004	4	TSEK	ND	Sau3A^{s}, MboI^{s}
6596	4/29/2004	4	T	ND	ND
6598	4/29/2004	4	TSEK	ND	ND
6615	5/4/2004	5	TE	ND	ND
6616	5/4/2004	5	TE	829	Sau3A^{s}, MboI^{s}
6618	5/4/2004	5	TSE	854	Sau3A^{s}, MboI^{s}
6620	5/4/2004	5	TE	1096	Sau3A^{s}, MboI^{r}
6621	5/4/2004	5	TS	ND	ND
6622	5/4/2004	5	TSE	1832	Sau3A^{s}, MboI^{s}
6624	5/4/2004	5	TSE	ND	ND
6625	5/4/2004	5	TSE	1151	Sau3A^{s}, MboI^{s}
6644	5/6/2004	6	TSE	ND	ND
6645	5/6/2004	6	TSK	854	Sau3A^{s}, MboI^{s}
6647	5/6/2004	6	TS	ND	ND
6648	5/6/2004	6	TSK	ND	ND
6649	5/6/2004	6	TK	1096	Sau3A^{s}, MboI^{r}
7025	6/23/2004	1	TS	ND	Sau3A^{s}, MboI^{s}
7026	6/23/2004	1	TE	ND	Sau3A^{s}, MboI^{s}
7033	6/23/2004	1	TE	ND	Sau3A^{s}, MboI^{r}
7034	6/23/2004	1	TSE	ND	Sau3A^{s}, MboI^{r}
7176	7/6/2004	4	TE	ND	Sau3A^{s}, MboI^{r}
7178	7/6/2004	4	TSE	ND	ND
7181	7/6/2004	4	TE	ND	Sau3A^{s}, MboI^{s}
7182	7/6/2004	4	TSE	ND	ND
7184	7/6/2004	4	TE	ND	ND
7187	7/6/2004	4	TE	ND	ND
7334	7/13/2004	1	TSE	ND	Sau3A^{s}, MboI^{s}
7336	7/13/2004	1	TE	ND	ND
7340	7/13/2004	1	TSE	ND	ND
7341	7/13/2004	1	TS	ND	Sau3A^{s}, MboI^{s}
7343	7/13/2004	1	TE	ND	ND
7346	7/13/2004	1	TSE	ND	ND
7348	7/13/2004	1	TE	ND	ND
7350	7/13/2004	1	TE	ND	ND
7351	7/13/2004	1	T	ND	ND
7354	7/13/2004	1	TSE	ND	Sau3A^{s}, MboI^{s}
7358	7/13/2004	1	TSE	ND	ND
7362	7/13/2004	1	TE	ND	Sau3A^{s}, MboI^{s}
7366	7/13/2004	1	T	ND	ND
7370	7/13/2004	1	SE	ND	Sau3A^{s}, MboI^{r}
7371	7/13/2004	1	TE	ND	ND
7372	7/13/2004	1	TSE	ND	ND
7830	8/5/2004	4	TSE	ND	ND

Appendix 6.2 continued

ID	Received	Farm	Resistance Profile	MLST	Methylation
7831	8/5/2004	4	TE	ND	Sau3A^{s}, MboI^{s}
7833	8/5/2004	4	TSE	ND	ND
7834	8/5/2004	4	TEK	ND	Sau3A^{s}, MboI^{s}
7836	8/5/2004	4	TE	ND	ND
7838	8/5/2004	4	TE	ND	ND
7840	8/5/2004	4	TSE	ND	Sau3A^{s}, MboI^{s}
7841	8/5/2004	4	TSEK	ND	ND
7941	8/11/2004	1	TE	ND	Sau3A^{s}, MboI^{s}
7943	8/11/2004	1	TE	ND	ND
7944	8/11/2004	1	TSE	ND	ND
7946	8/11/2004	1	TSE	ND	ND
7950	8/11/2004	1	TE	ND	Sau3A^{s}, MboI^{s}
7951	8/11/2004	1	T	ND	ND
7953	8/11/2004	1	TSE	ND	ND
7955	8/11/2004	1	SE	ND	Sau3A^{s}, MboI^{r}
7957	8/11/2004	1	TE	ND	ND
7961	8/11/2004	1	TSE	ND	ND
7963	8/11/2004	1	TSE	ND	ND
7965	8/11/2004	1	TSE	ND	ND
7969	8/11/2004	1	TSE	ND	ND
7974	8/11/2004	1	TSE	ND	Sau3A^{s}, MboI^{r}
7976	8/11/2004	1	TSE	ND	ND
7977	8/11/2004	1	TEK	ND	Sau3A^{s}, MboI^{s}
8107	8/20/2004	4	TSEK	ND	ND
8108	8/20/2004	4	TSE	ND	Sau3A^{s}, MboI^{r}
8109	8/20/2004	4	TE	ND	ND
8110	8/20/2004	4	TSE	ND	ND
8112	8/20/2004	4	TSE	ND	Sau3A^{s}, MboI^{r}
8114	8/20/2004	4	TE	ND	ND
8116	8/20/2004	4	TSE	ND	ND
8117	8/20/2004	4	TEK	ND	ND
8118	8/20/2004	4	TE	ND	ND
8415	9/22/2004	7	TSE	ND	ND
8417	9/22/2004	7	TSE	ND	ND
8419	9/22/2004	7	TSE	ND	ND
8420	9/22/2004	7	TSEK	ND	ND
8425	9/22/2004	7	TSE	1834	Sau3A^{s}, MboI^{s}
8426	9/22/2004	7	TE	ND	ND
8430	9/22/2004	7	TSE	ND	ND
8432	9/22/2004	7	TSE	ND	ND
8435	9/22/2004	7	TE	ND	ND
8436	9/22/2004	7	TSE	ND	ND
8442	9/22/2004	7	TSE	ND	ND
8445	9/22/2004	7	TE	890	ND
8446	9/22/2004	7	TSE	ND	ND
8450	9/22/2004	7	TSE	ND	ND
8678	10/6/2004	7	TEK	ND	ND
8679	10/6/2004	7	TE	ND	ND
8683	10/6/2004	7	TE	ND	ND
8685	10/6/2004	7	TE	ND	ND
8687	10/6/2004	7	TSEK	ND	ND
8689	10/6/2004	7	TSE	1109	Sau3A^{s}, MboI^{r}

Appendix 6.2 continued

ID	Received	Farm	Resistance Profile	MLST	Methylation
8690	10/6/2004	7	TE	1836	Sau3A^{s}, MboI^{s}
8694	10/6/2004	7	TE	ND	ND
8697	10/6/2004	7	TE	1837	Sau3A^{s}, MboI^{s}
8700	10/6/2004	7	TSE	ND	ND
8708	10/6/2004	7	TSE	ND	ND
8710	10/6/2004	7	TE	1096	Sau3A^{s}, MboI^{r}
8711	10/6/2004	7	TSE	ND	ND
8712	10/6/2004	7	TE	ND	ND
8714	10/6/2004	7	TE	ND	ND
8716	10/6/2004	7	TE	ND	ND
8910	10/27/2004	7	TSE	ND	ND
8913	10/27/2004	7	TSE	ND	ND
8915	10/27/2004	7	TSE	1545	Sau3A^{s}, MboI^{s}
8918	10/27/2004	7	TSE	ND	ND
8920	10/27/2004	7	TEK	ND	ND
8921	10/27/2004	7	TSE	ND	ND
8923	10/27/2004	7	TE	829	Sau3A^{s}, MboI^{s}
8924	10/27/2004	7	TSE	ND	ND
8925	10/27/2004	7	TEK	ND	ND
8927	10/27/2004	7	TEK	1157	Sau3A^{s}, MboI^{s}
8929	10/27/2004	7	TE	ND	ND
8933	10/27/2004	7	TSE	ND	ND
8936	10/27/2004	7	TE	ND	ND
8939	10/27/2004	7	TSE	ND	ND
8941	10/27/2004	7	TSE	ND	ND
8943	10/27/2004	7	TSE	854	ND
8945	10/27/2004	7	TE	ND	ND
8948	10/27/2004	7	TE	ND	ND
9079	11/17/2004	7	TE	ND	ND
9080	11/17/2004	7	TSE	ND	ND
9084	11/17/2004	7	TE	ND	ND
9085	11/17/2004	7	TSE	1109	ND
9088	11/17/2004	7	TE	ND	ND
9090	11/17/2004	7	TE	ND	ND
9091	11/17/2044	7	TE	1836	ND
9092	11/17/2004	7	TE	ND	ND
9093	11/17/2004	7	TSE	854	Sau3A^{s}, MboI^{s}
9095	11/17/2004	7	TSE	ND	Sau3A^{s}, MboI^{r}
9096	11/17/2004	7	TE	ND	ND
9100	11/17/2004	7	TSE	ND	ND
9103	11/17/2004	7	TE	ND	ND
9104	11/17/2004	7	TSE	1151	Sau3A^{s}, MboI^{s}
9107	11/17/2004	7	TSE	ND	ND
9108	11/17/2004	7	TSE	ND	ND
9110	11/17/2004	7	TSE	ND	ND
9111	11/17/2004	7	TEK	ND	ND
9113	11/17/2004	7	TSE	ND	ND
9116	11/17/2004	7	TSE	ND	ND
9547	1/19/2005	7	TSE	ND	ND
9549	1/19/2005	7	TSE	ND	ND
9550	1/19/2005	7	TE	ND	ND
9553	1/19/2005	7	TSE	ND	ND

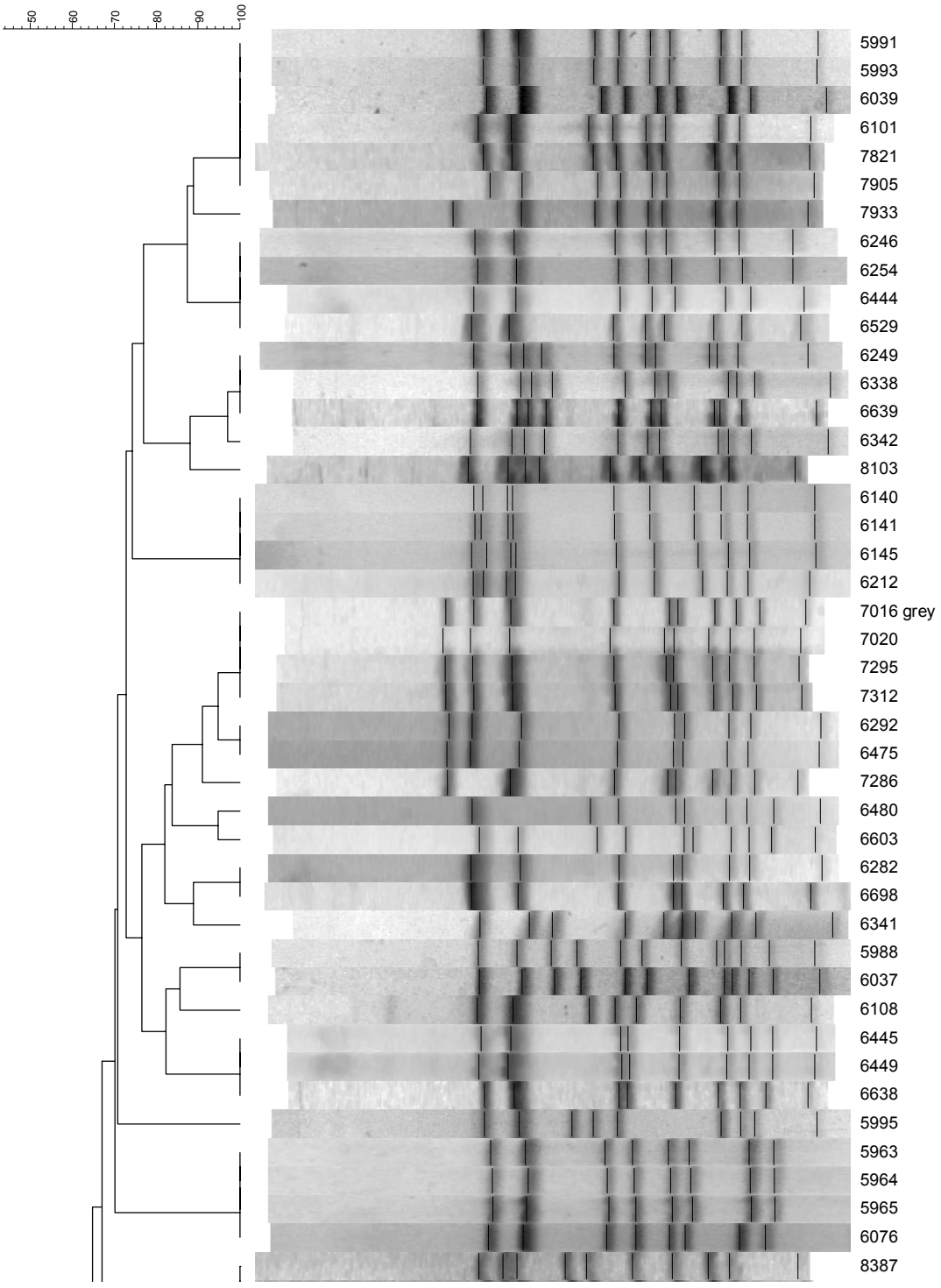
Appendix 6.2 continued

ID	Received	Farm	Resistance Profile	MLST	Methylation
9554	1/19/2005	7	TE	ND	ND
9558	1/19/2005	7	TSE	ND	ND
9560	1/19/2005	7	TE	ND	ND
9563	1/19/2005	7	TSE	ND	ND
9569	1/19/2005	7	TSE	ND	ND
9571	1/19/2005	7	TE	ND	ND
9573	1/19/2005	7	TSE	ND	ND
9574	1/19/2005	7	TE	ND	ND
9576	1/19/2005	7	TSE	ND	ND
9578	1/19/2005	7	TE	ND	ND
9580	1/19/2005	7	TE	ND	ND
9584	1/19/2005	7	TE	ND	ND
9629	2/1/2005	7	TE	ND	ND
9633	2/1/2005	7	TE	ND	ND
9635	2/1/2005	7	TSE	ND	ND
9637	2/1/2005	7	TSE	ND	ND
9641	2/1/2005	7	TE	ND	ND
9644	2/1/2005	7	TSE	ND	ND
9649	2/1/2005	7	TSE	ND	ND
9650	2/1/2005	7	TE	ND	ND
9654	2/1/2005	7	TSE	ND	ND
9656	2/1/2005	7	TE	ND	ND
9658	2/1/2005	7	TSE	ND	ND
9660	2/1/2005	7	TSE	ND	ND
9664	2/1/2005	7	TE	ND	ND
9665	2/1/2005	7	TSE	ND	ND
9668	2/1/2005	7	TSE	ND	ND
9882	2/24/2005	7	TSE	ND	ND
9884	2/24/2005	7	TSE	ND	ND
9885	2/24/2005	7	TE	ND	ND
9887	2/24/2005	7	TE	ND	ND
9888	2/24/2005	7	TSEK	ND	ND
9890	2/24/2005	7	TE	ND	ND
9892	2/24/2005	7	TE	ND	ND
9894	2/24/2005	7	TE	ND	ND
9897	2/24/2005	7	TSE	ND	ND
9899	2/24/2005	7	TE	ND	ND
9901	2/24/2005	7	TSE	ND	ND
9903	2/24/2005	7	TE	ND	ND
9906	2/24/2005	7	TE	ND	ND
9914	2/24/2005	7	TE	ND	ND
9917	2/24/2005	7	TE	ND	ND
9920	2/24/2005	7	TSE	ND	ND
9965	3/18/2005	7	TSE	ND	ND
9967	3/18/2005	7	TE	ND	ND
9970	3/18/2005	7	TSE	ND	ND
9972	3/18/2005	7	TSE	ND	ND
9973	3/18/2005	7	TE	ND	ND
9975	3/18/2005	7	TE	ND	ND
9978	3/18/2005	7	TE	ND	ND
9979	3/18/2005	7	TSE	ND	ND
9982	3/18/2005	7	TSE	ND	ND

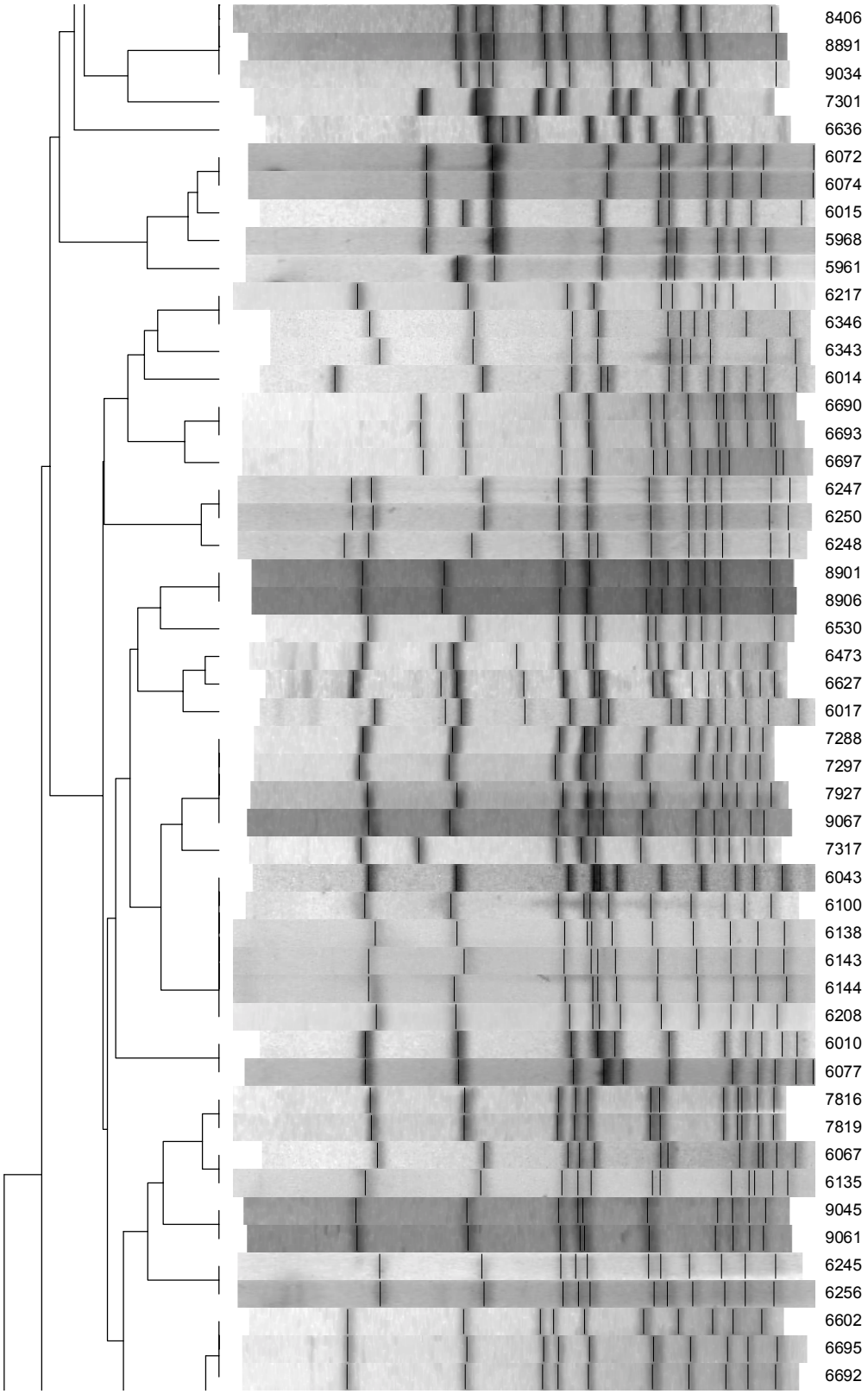
Appendix 6.2 continued

ID	Received	Farm	Resistance Profile	MLST	Methylation
9983	3/18/2005	7	TE	ND	ND
9985	3/18/2005	7	TSE	ND	ND
9989	3/18/2005	7	TSE	ND	ND
9994	3/18/2005	7	TSE	ND	ND
9996	3/18/2005	7	TE	ND	ND
10000	3/18/2005	7	TSE	ND	ND
10002	3/18/2005	7	TE	ND	ND

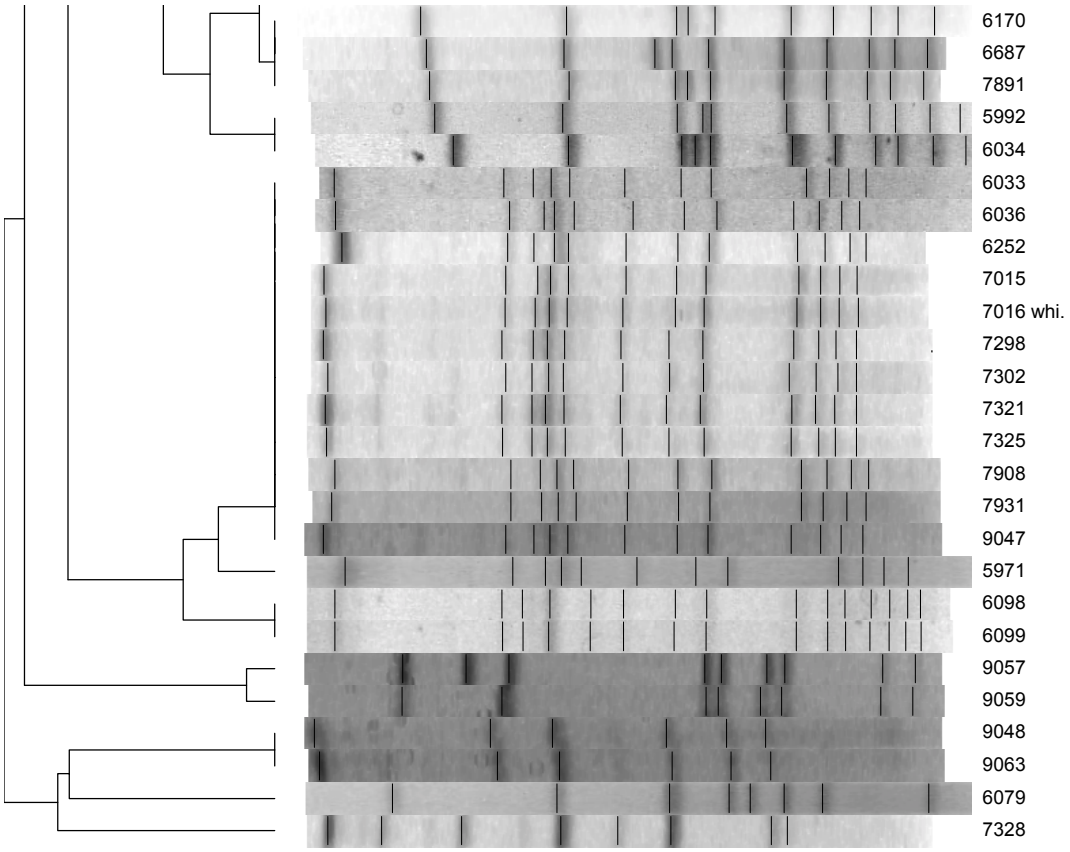
Appendix 6.3 PFGE analysis of turkey-derived *C. coli* strains using *Sma*I



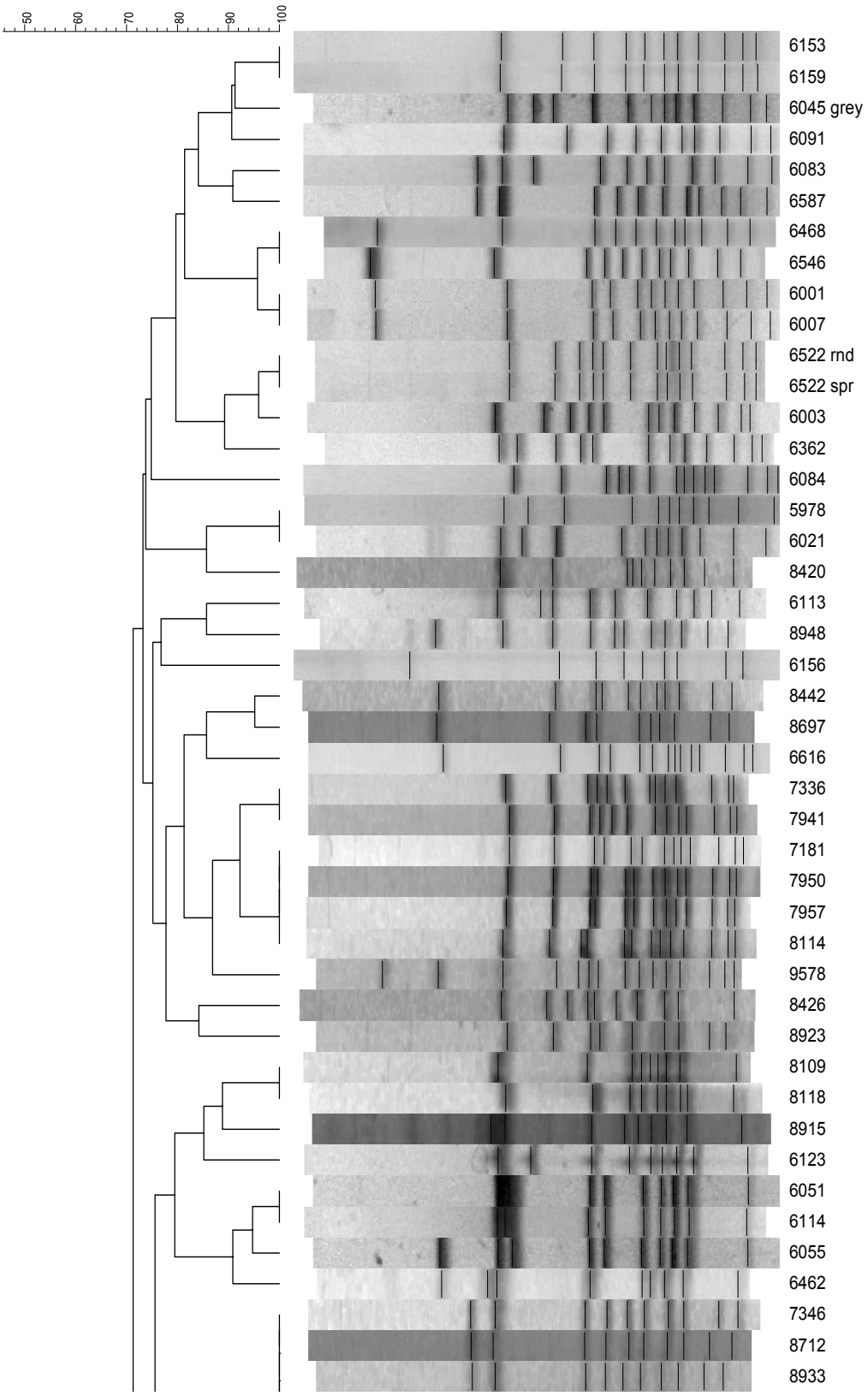
Appendix 6.3 continued



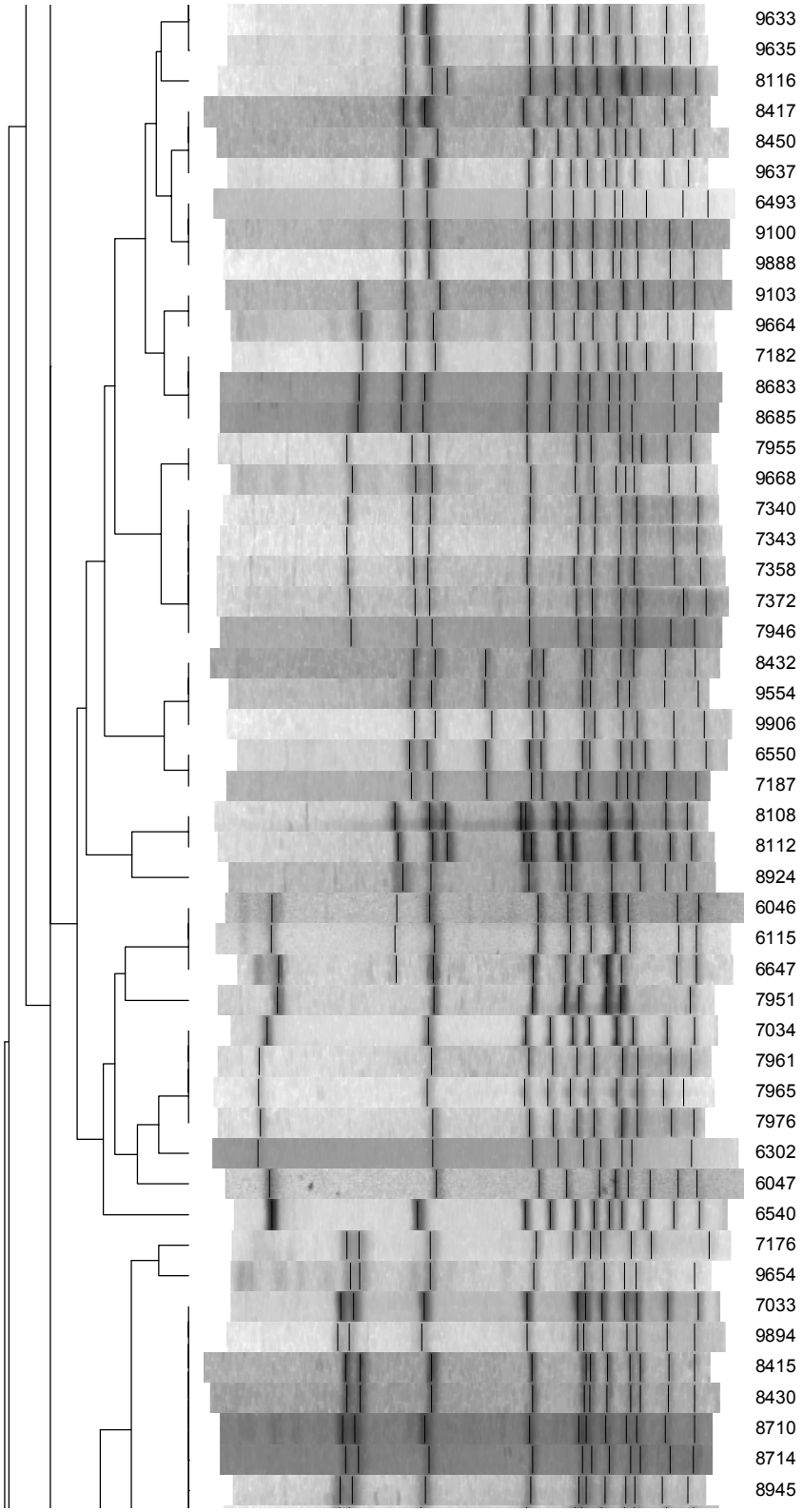
Appendix 6.3 continued



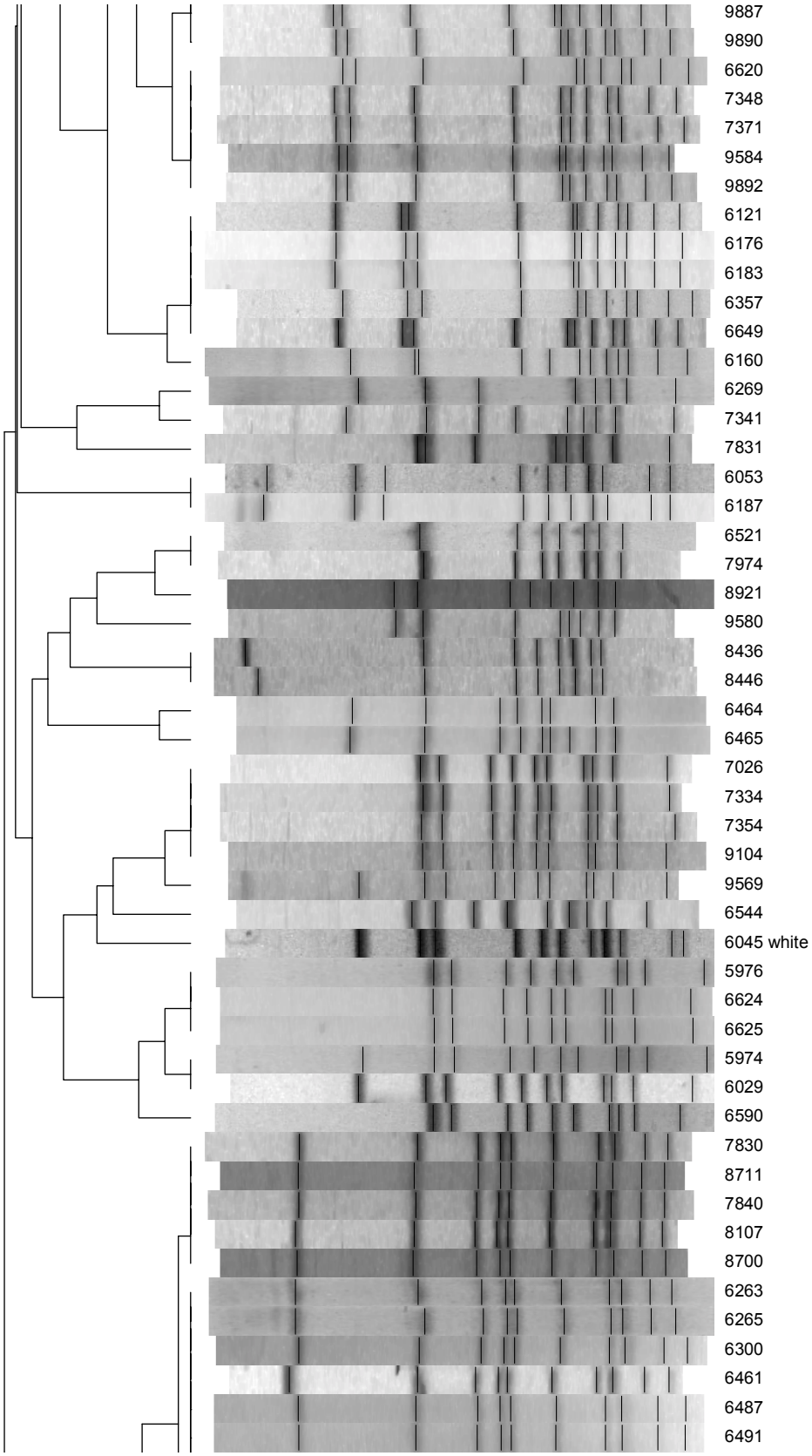
Appendix 6.4 PFGE analysis of swine-derived *C. coli* strains using *Sma*I



Appendix 6.4 continued



Appendix 6.4 continued



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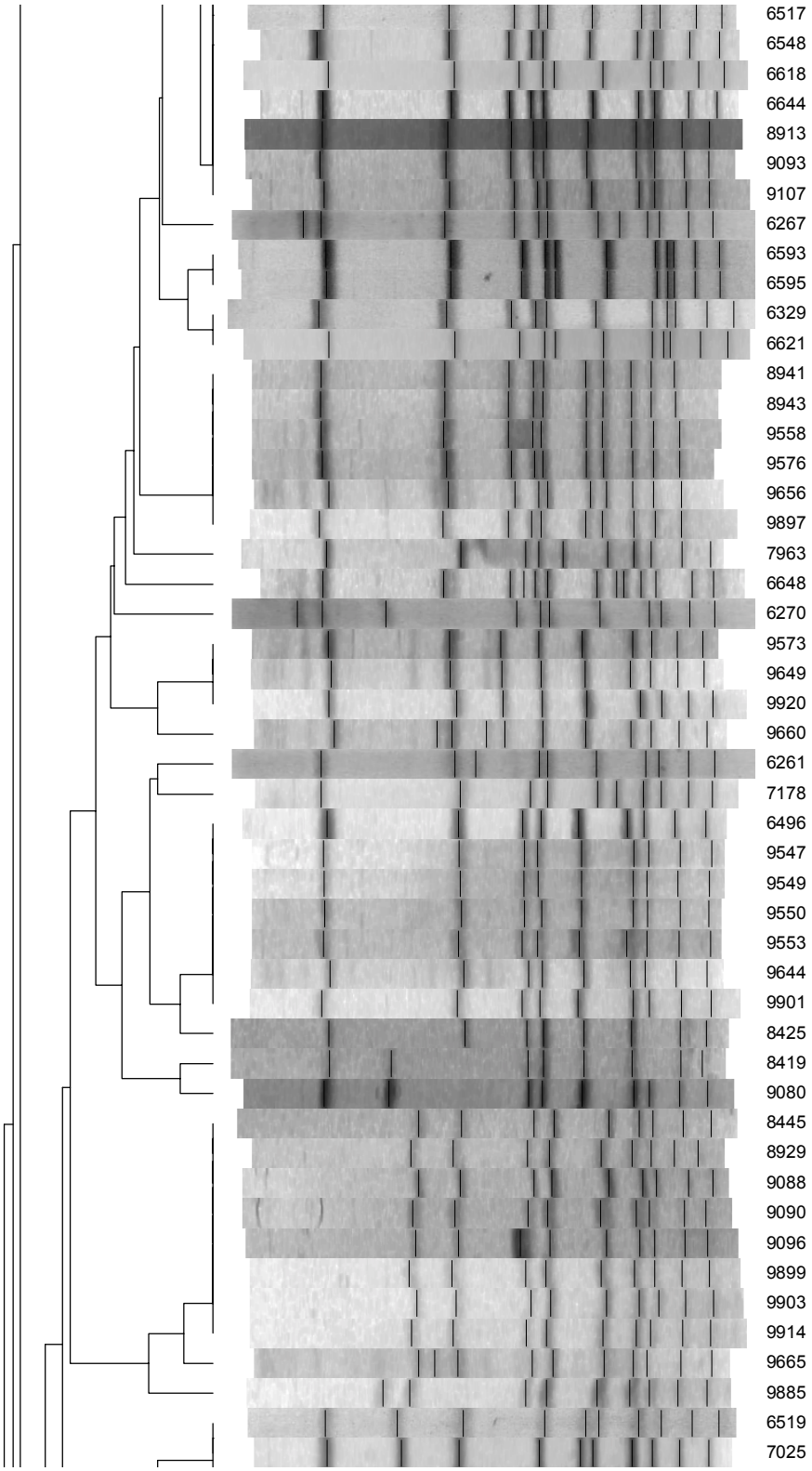

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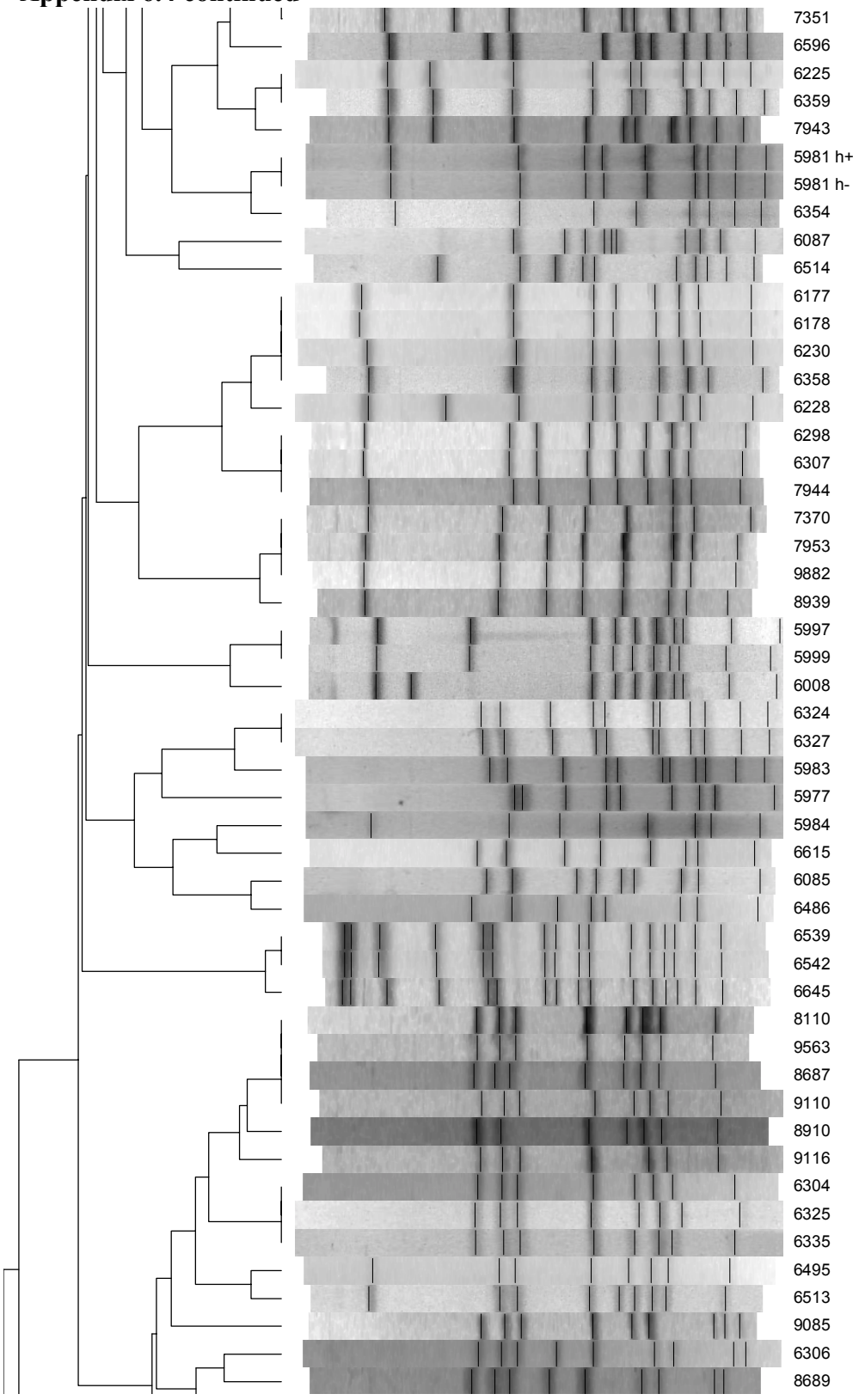

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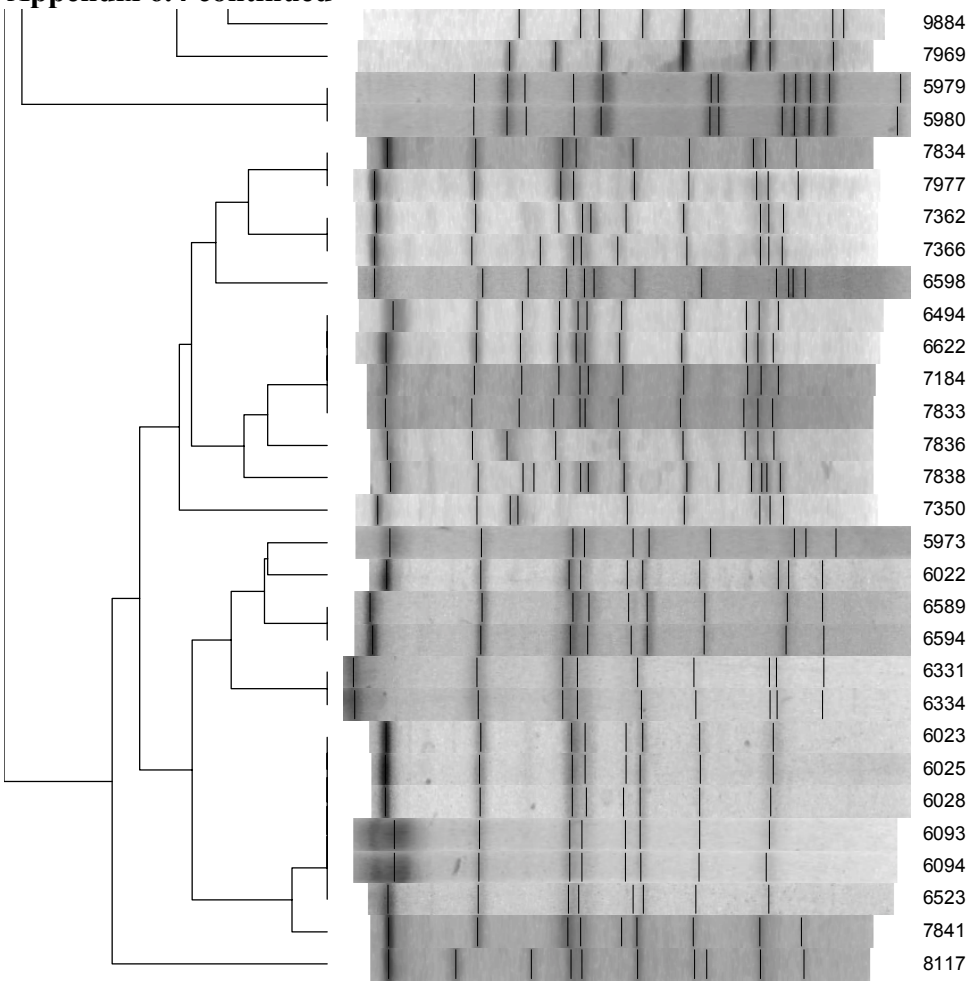
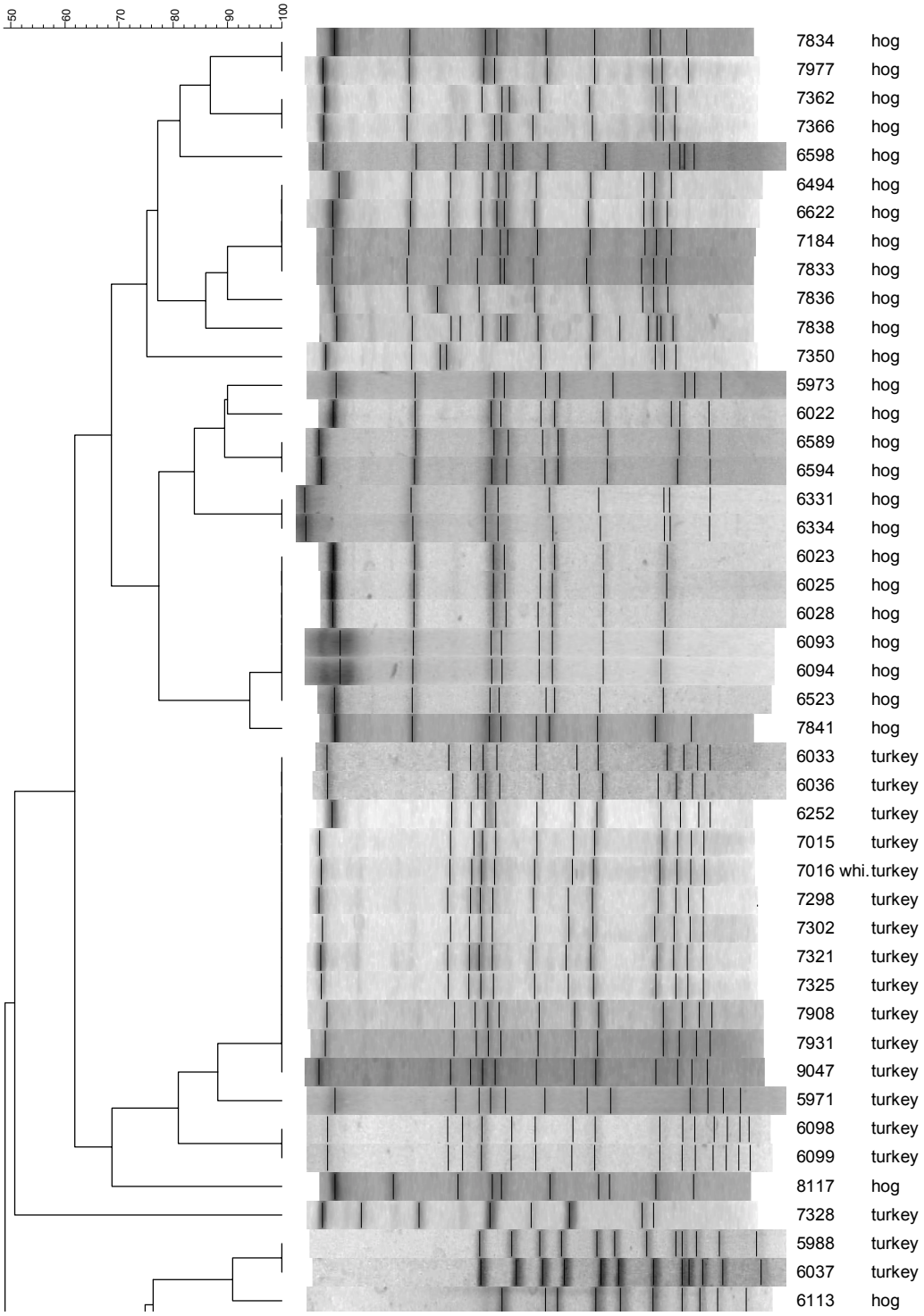


Appendix 6.5 PFGE analysis of turkey- and swine-derived *C. coli* strains using *Sma*I



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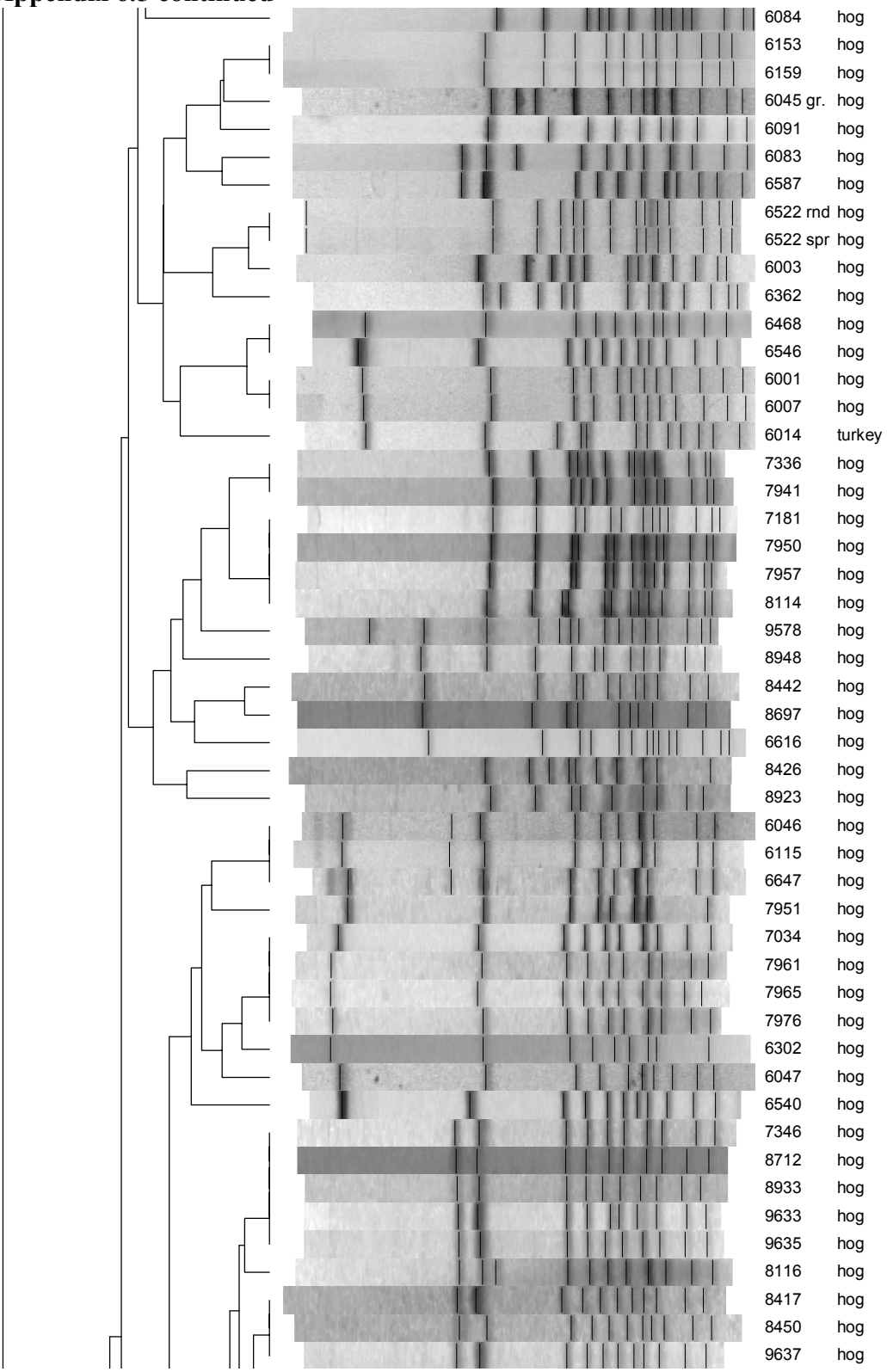

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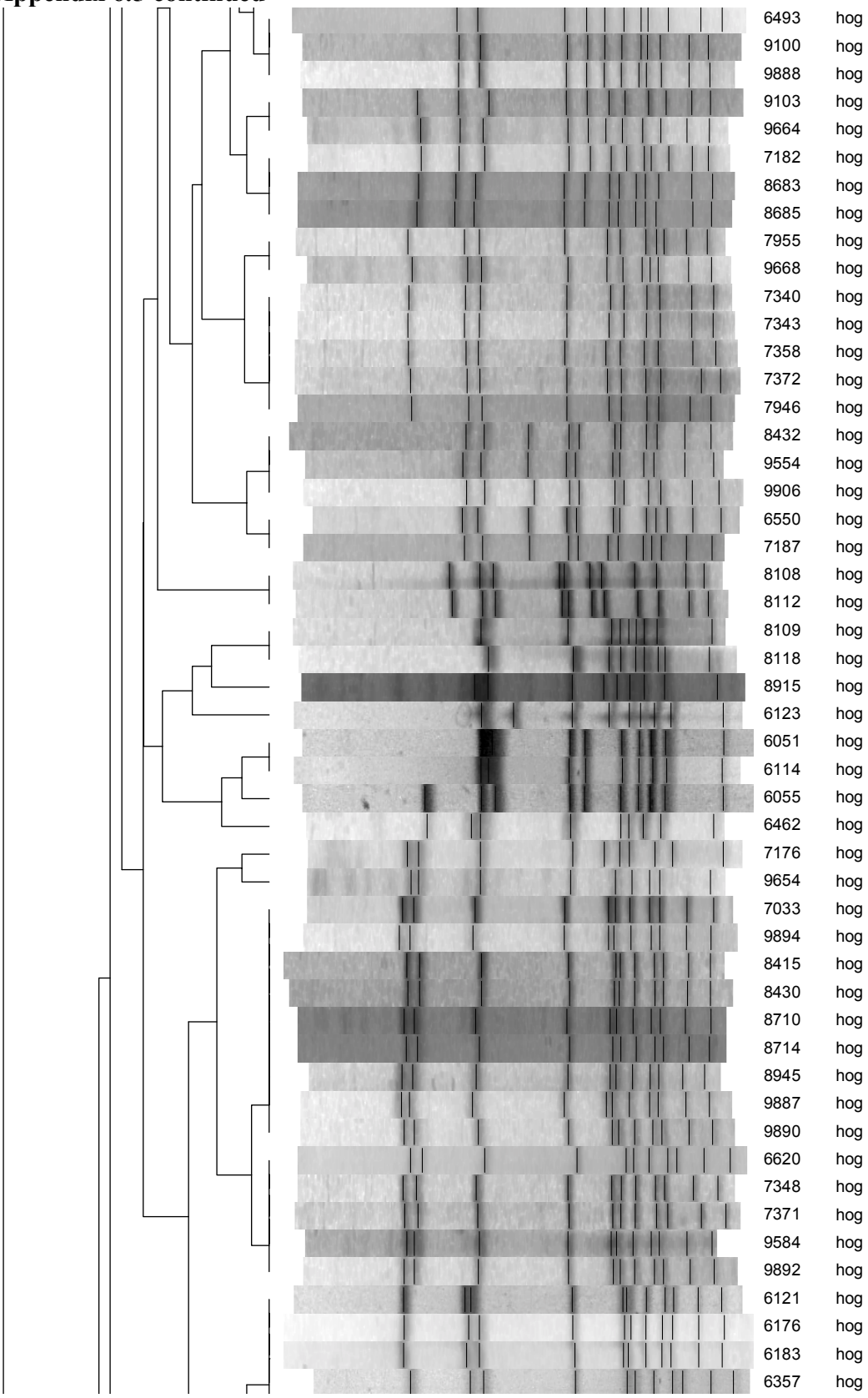

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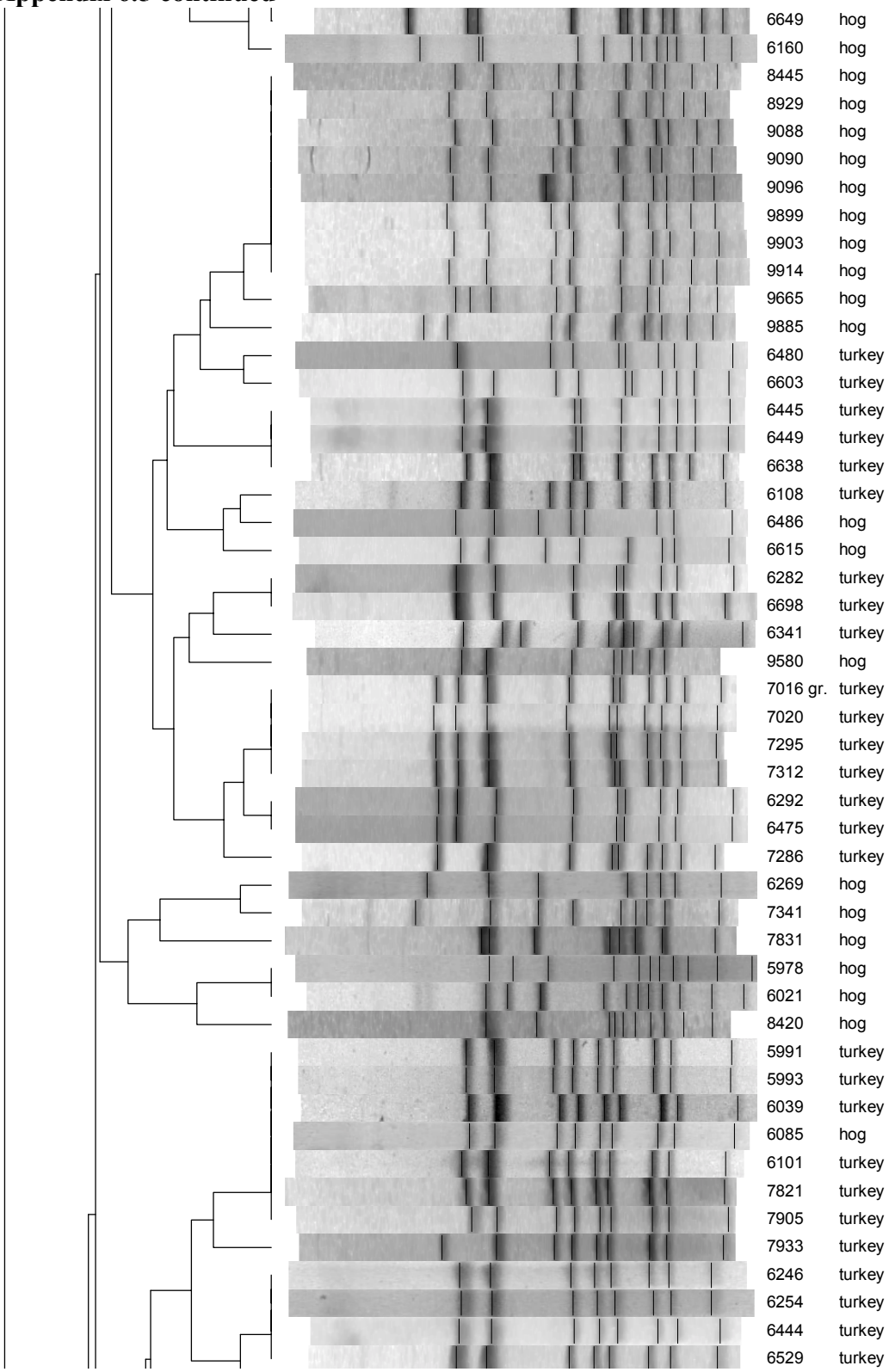

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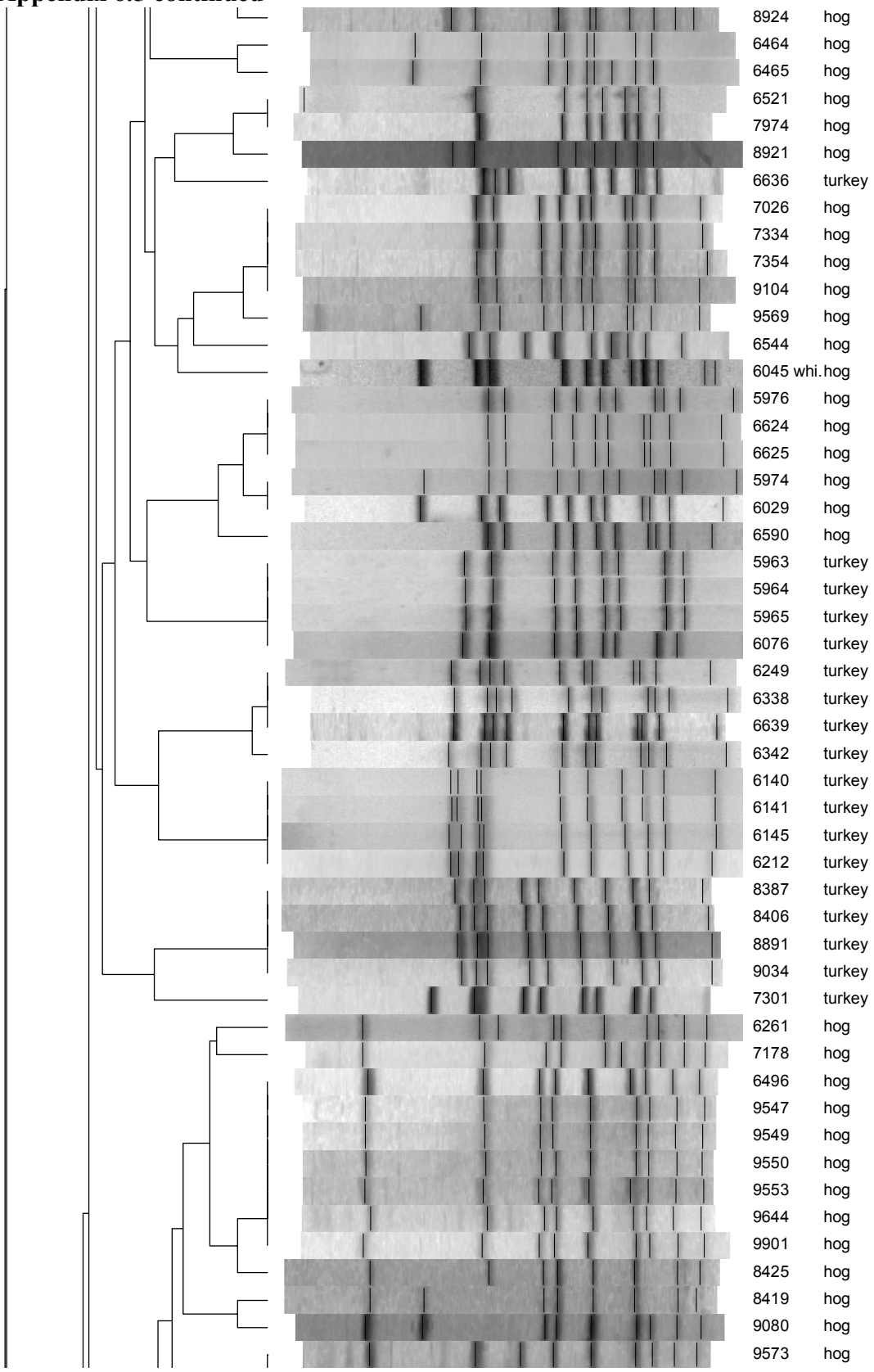

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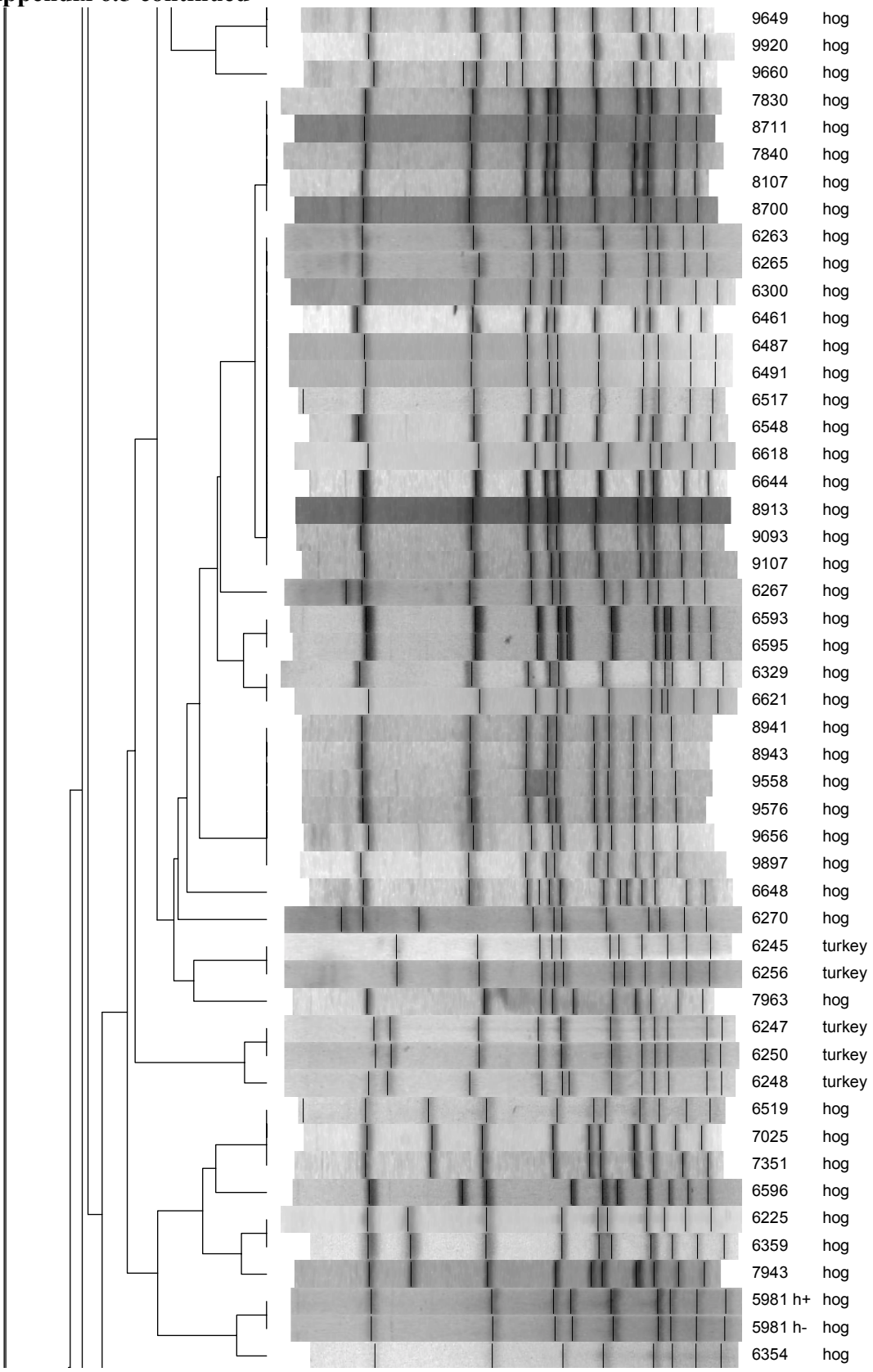

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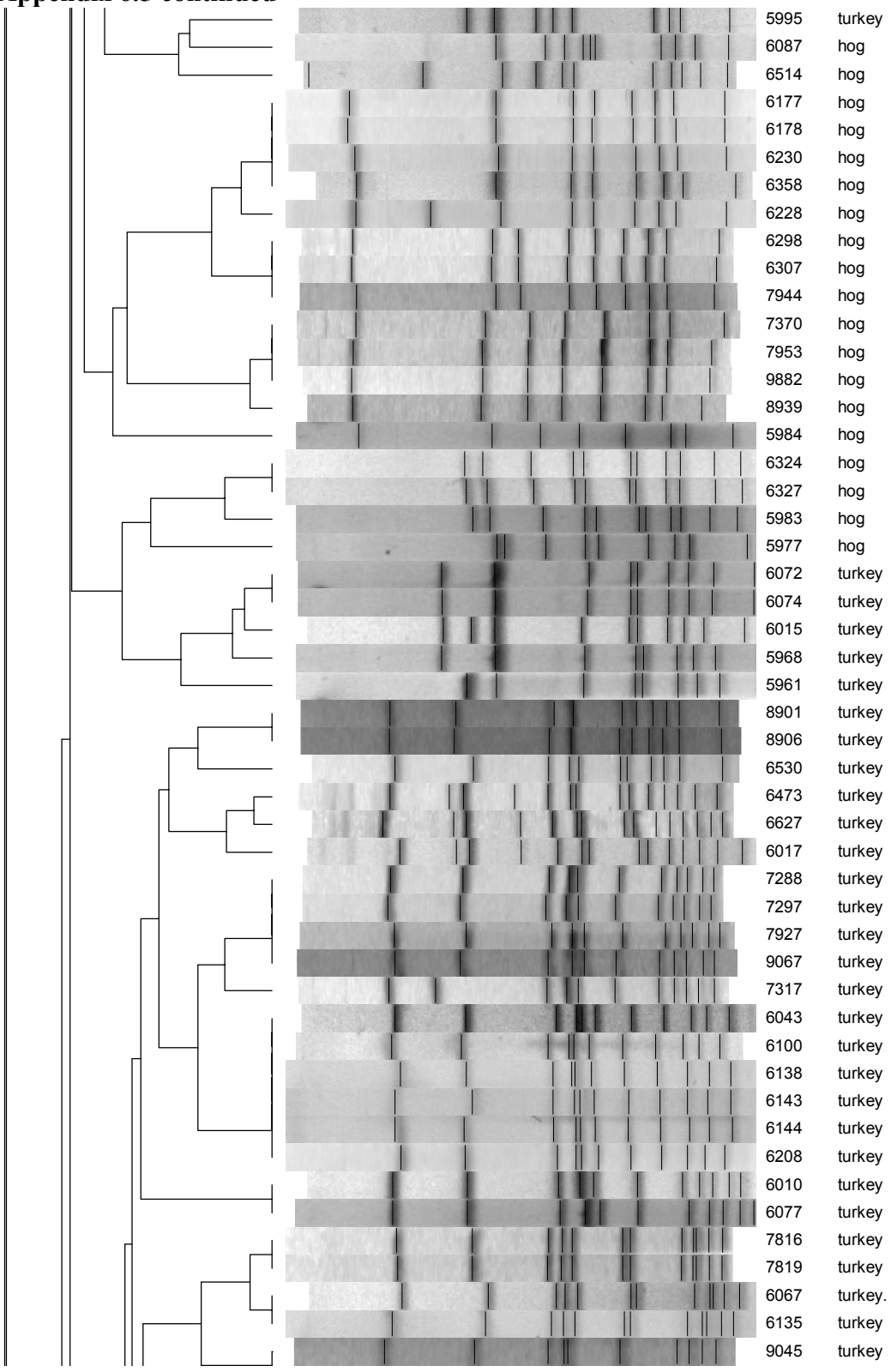

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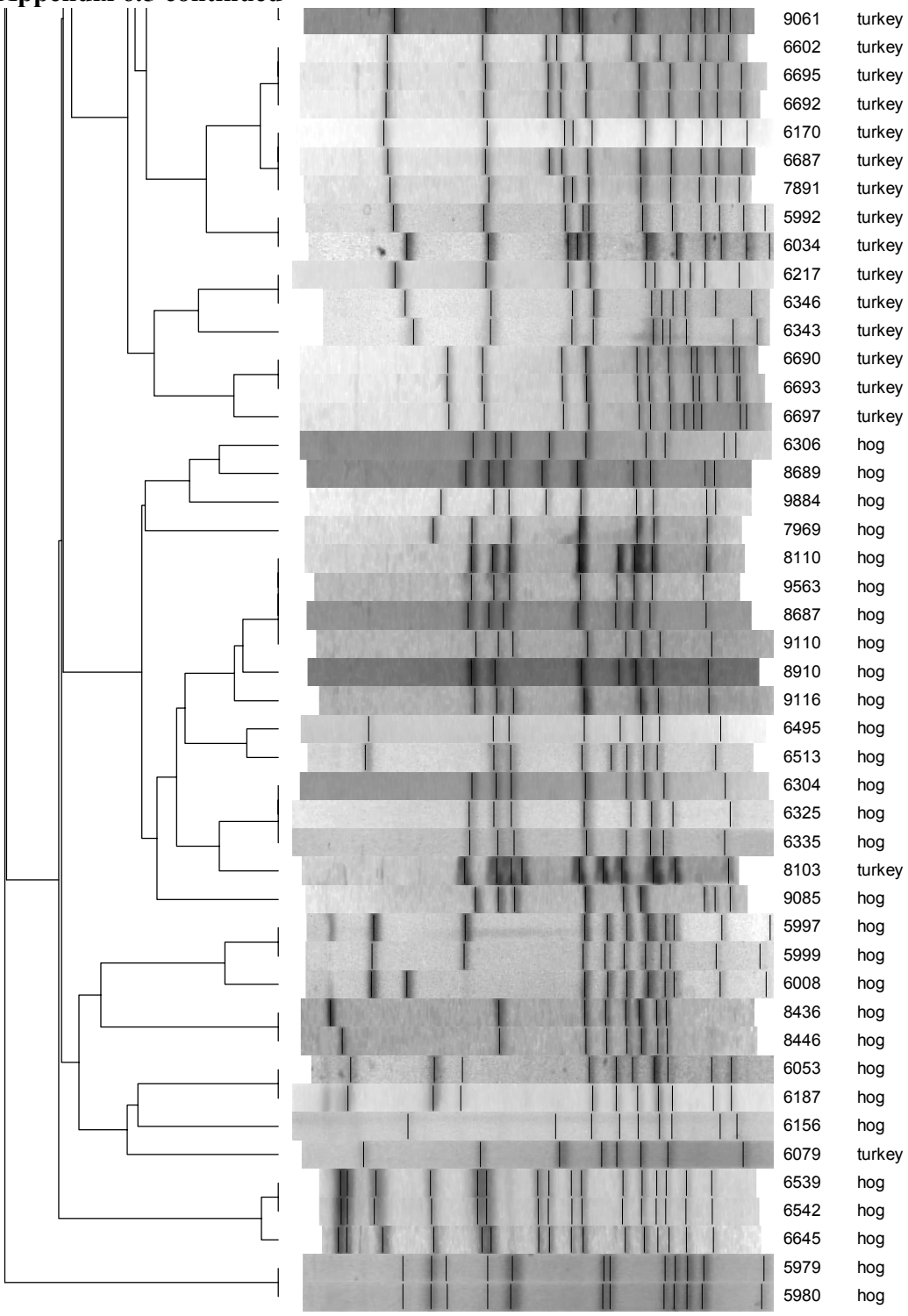
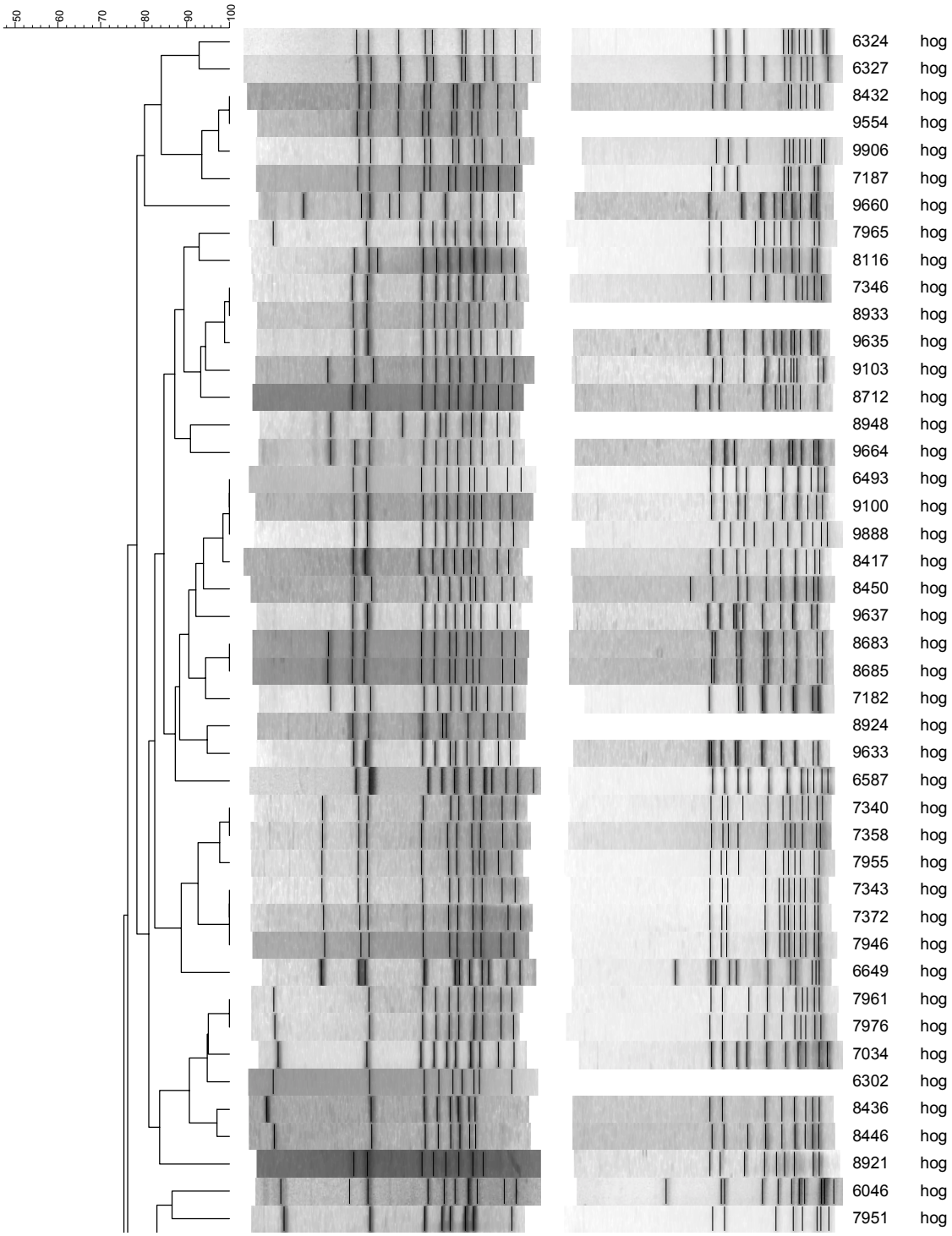


Appendix 6.6 PFGE analysis of turkey- and swine-derived *C. coli* strains using *Sma*I and *Kpn*I



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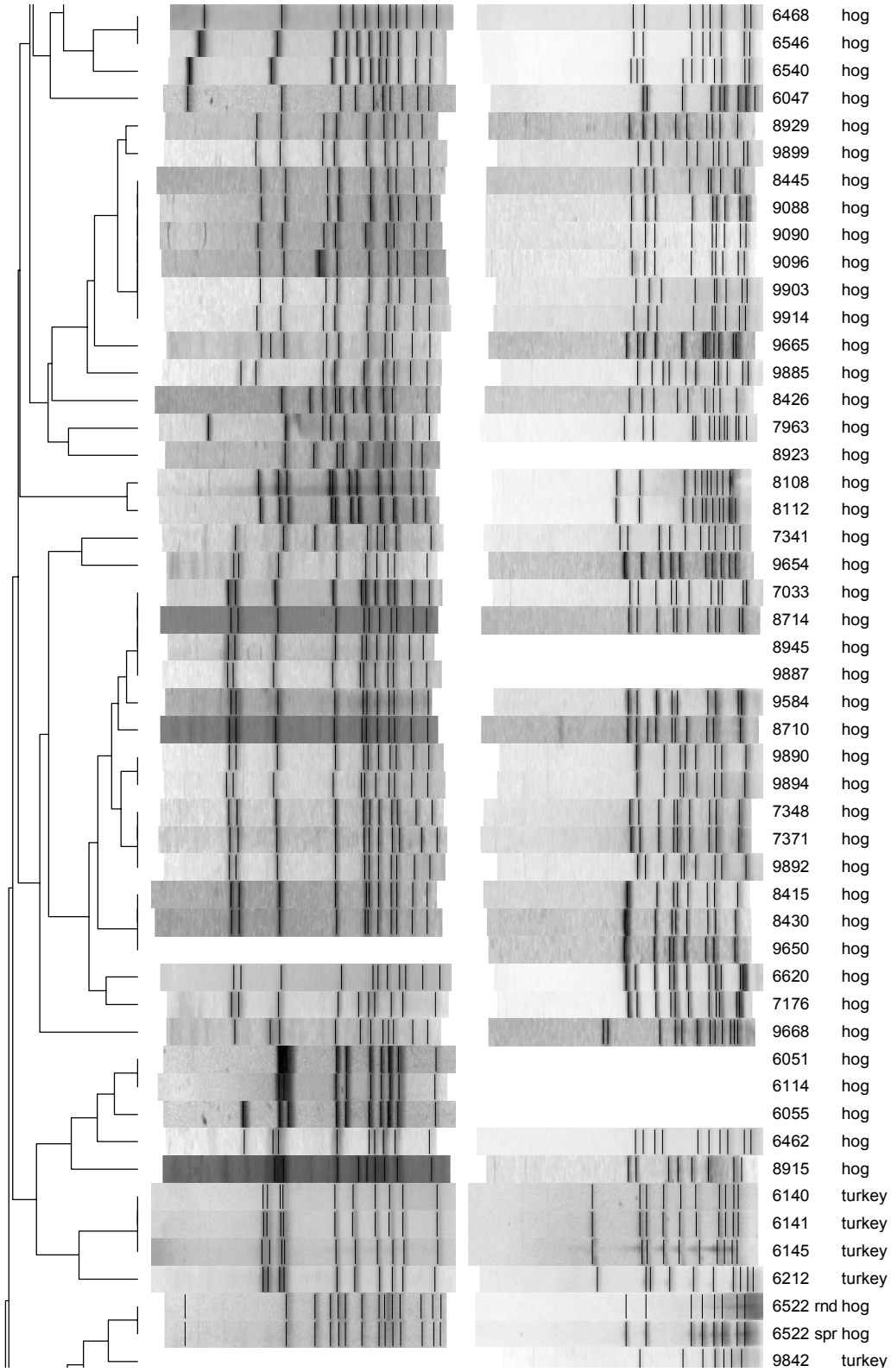

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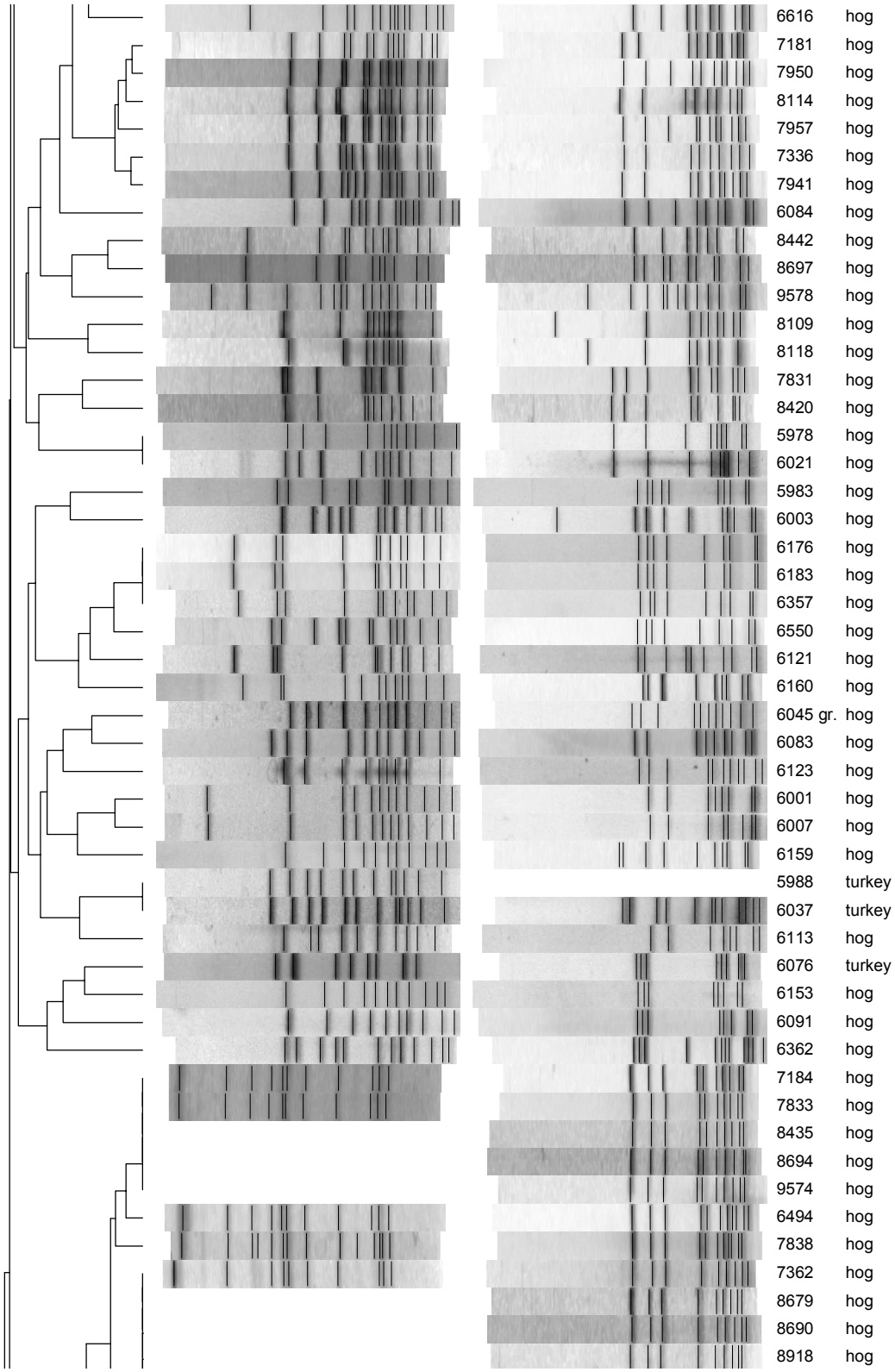

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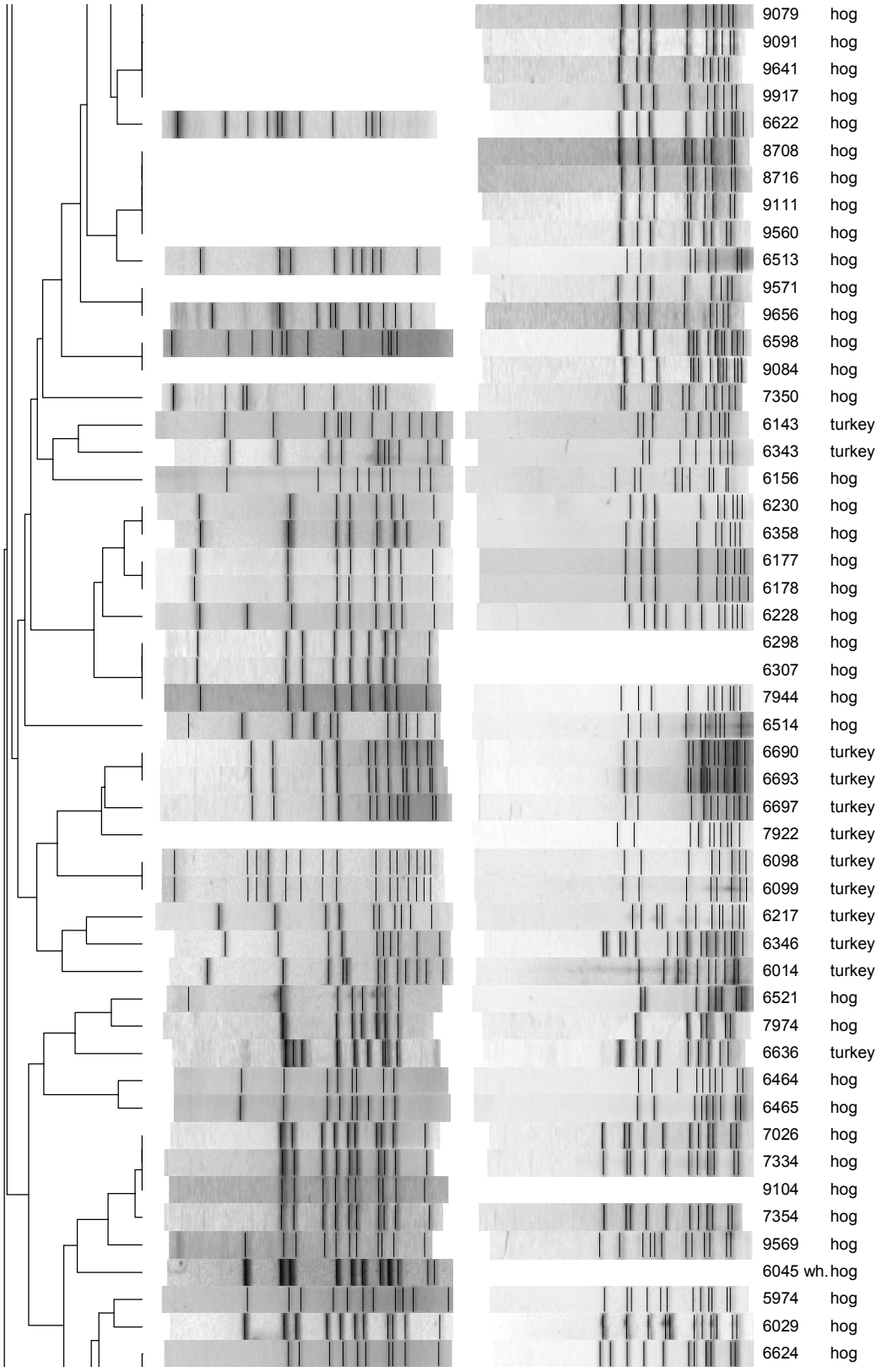

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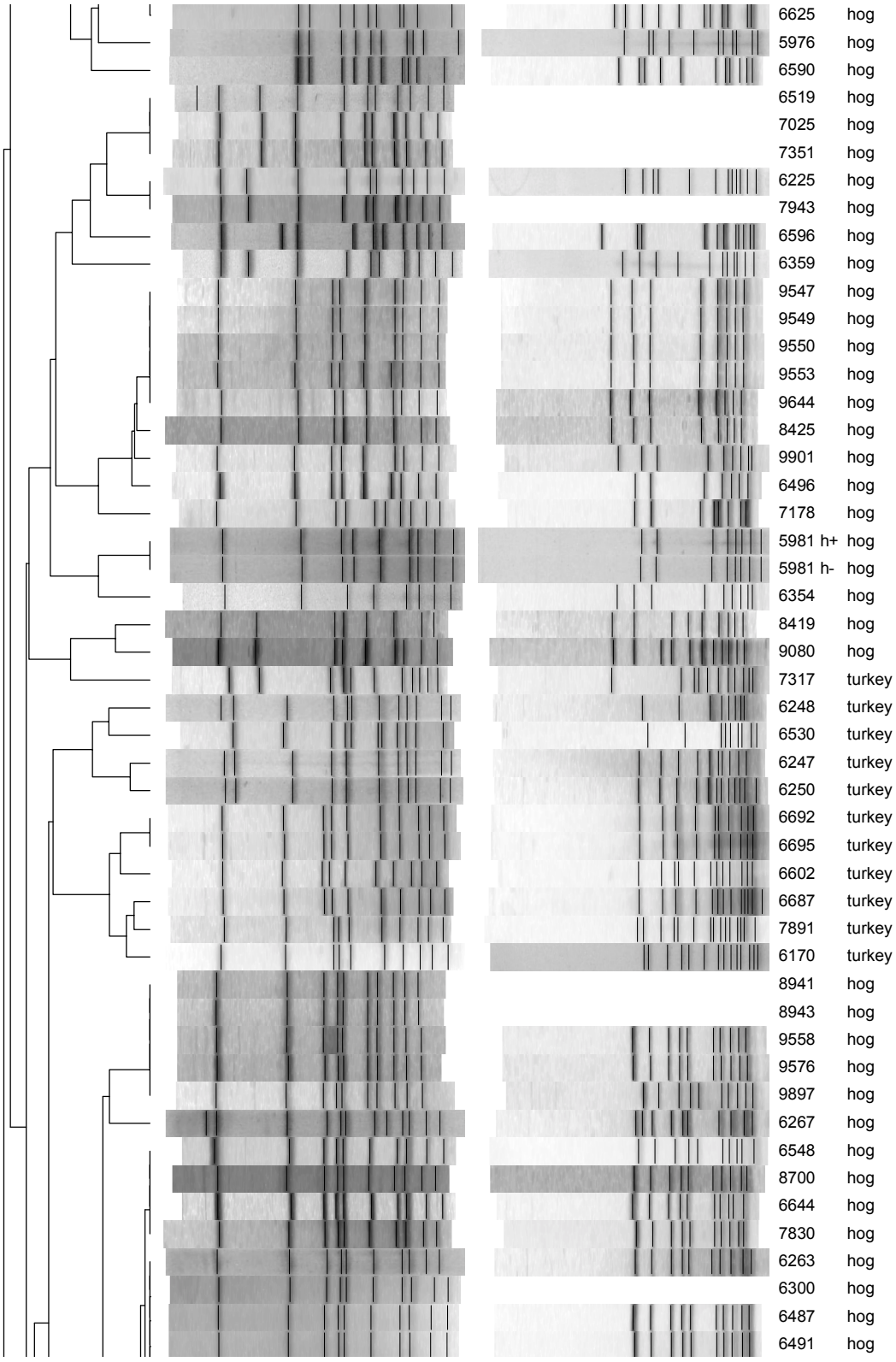

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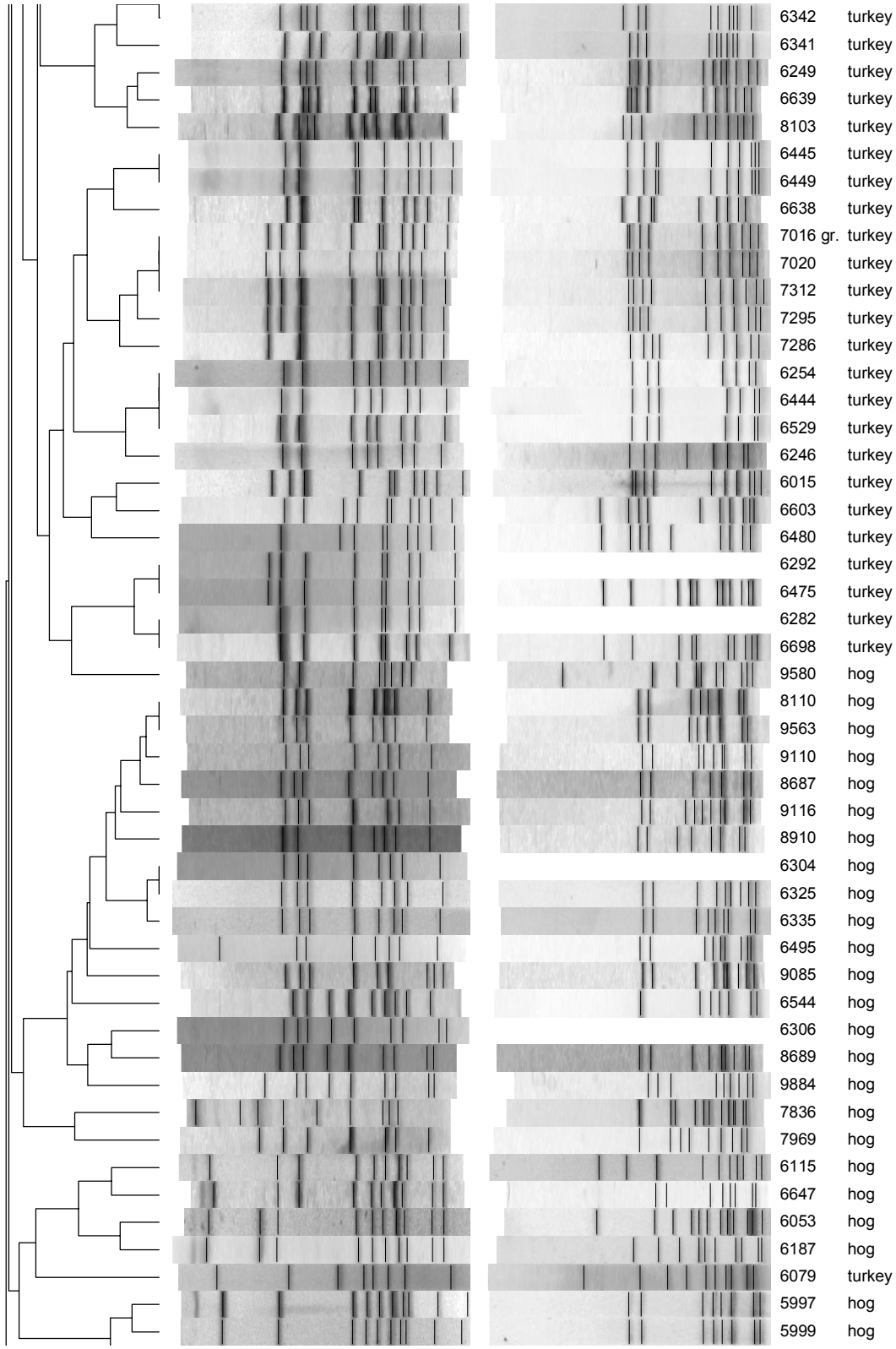

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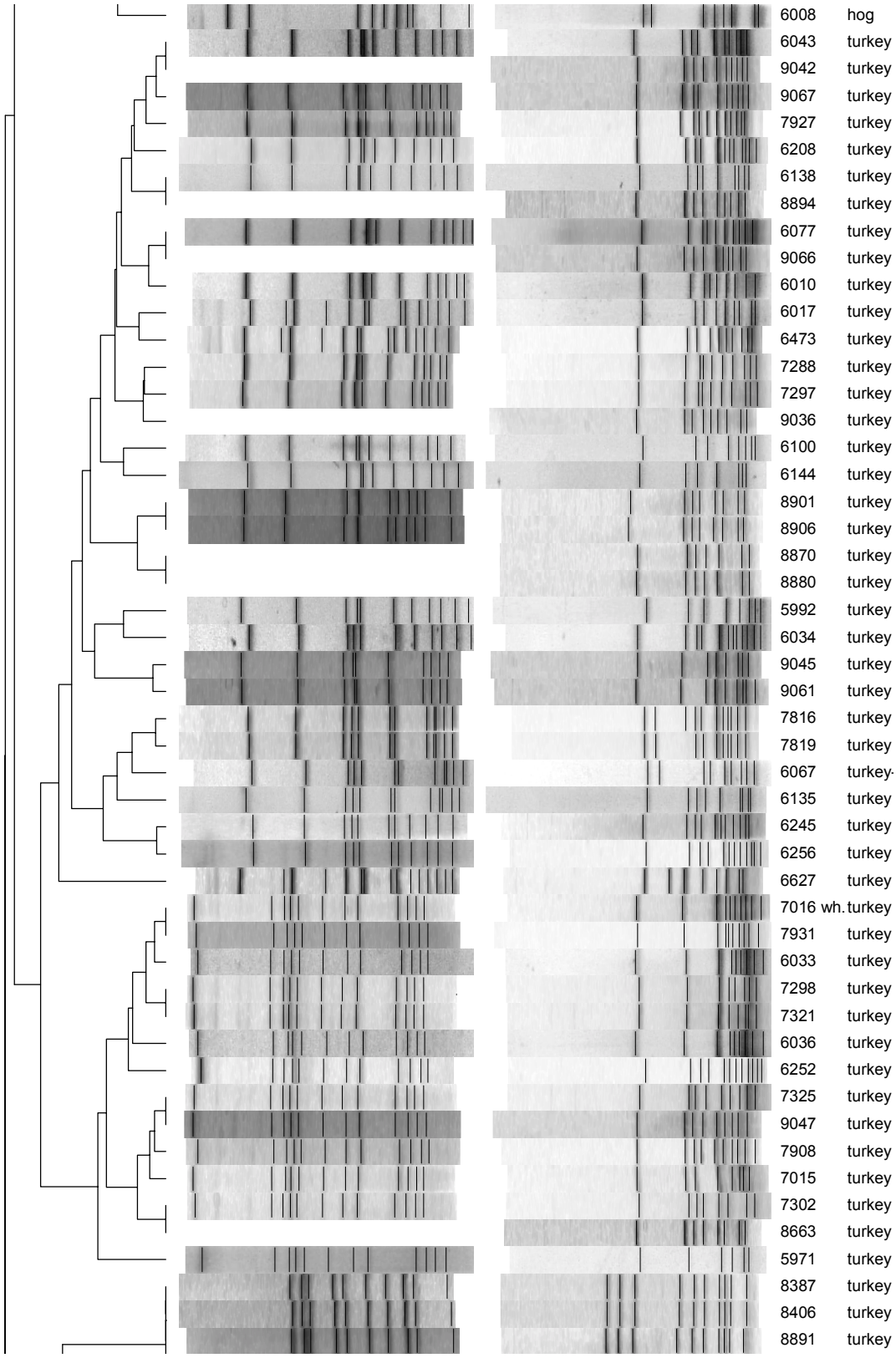

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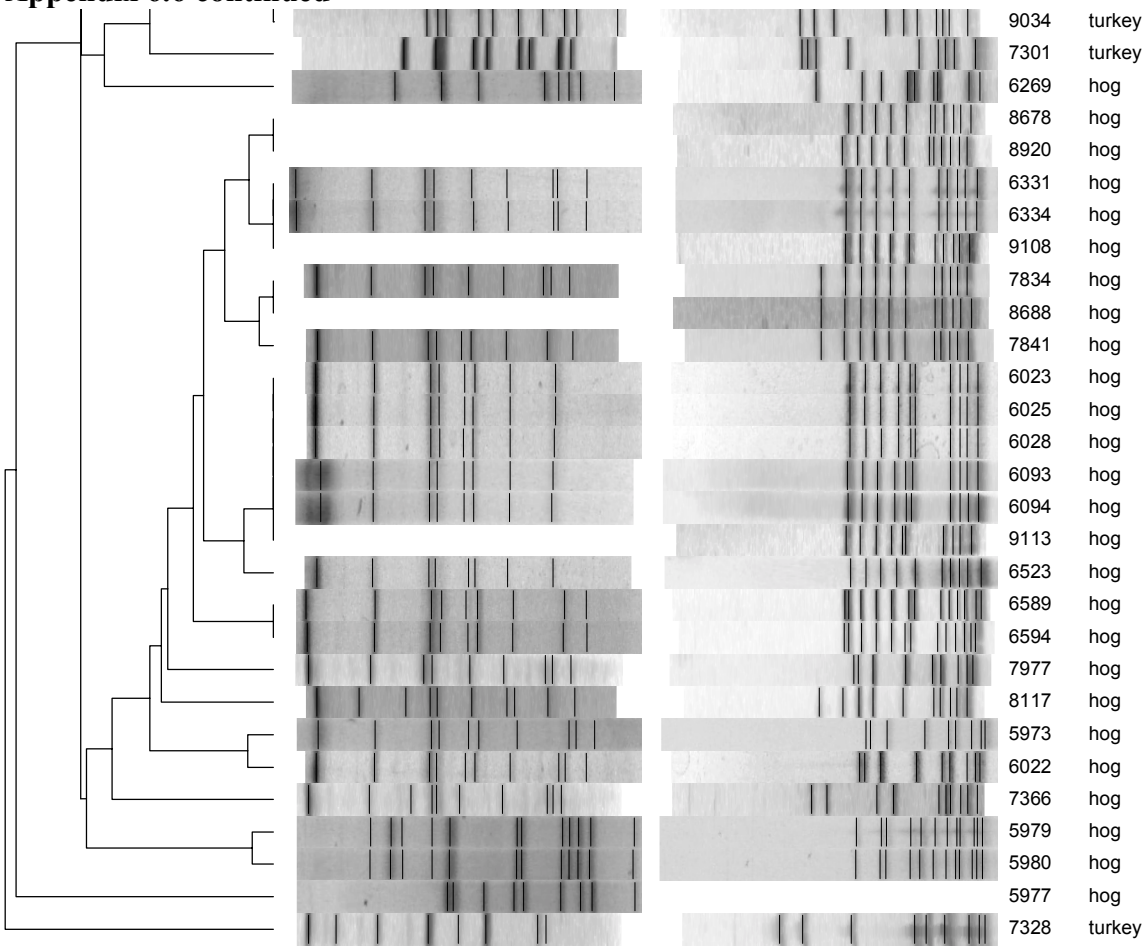
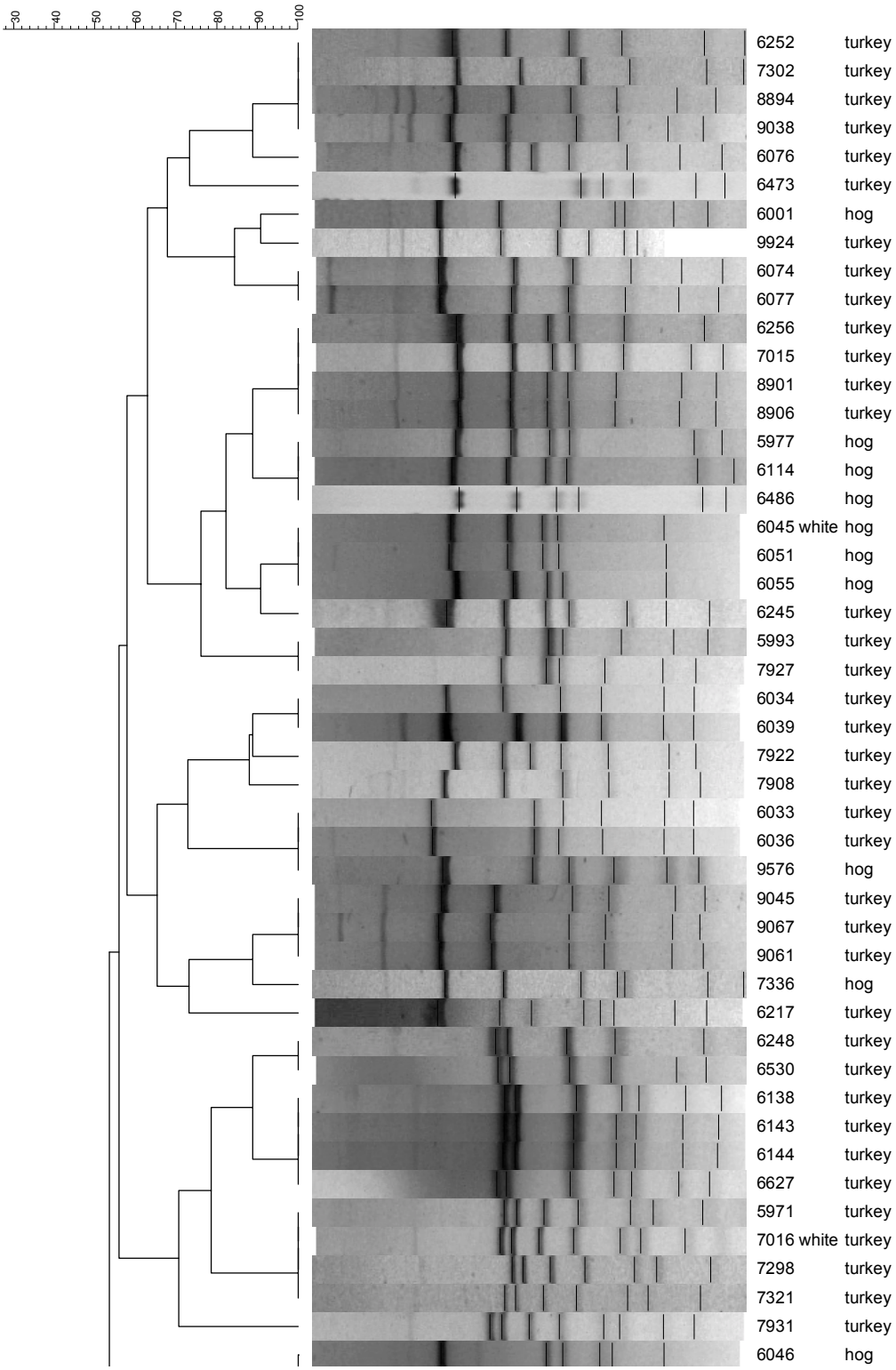


Appendix 6.7 *fla* analysis of turkey- and swine-derived *C. coli* strains



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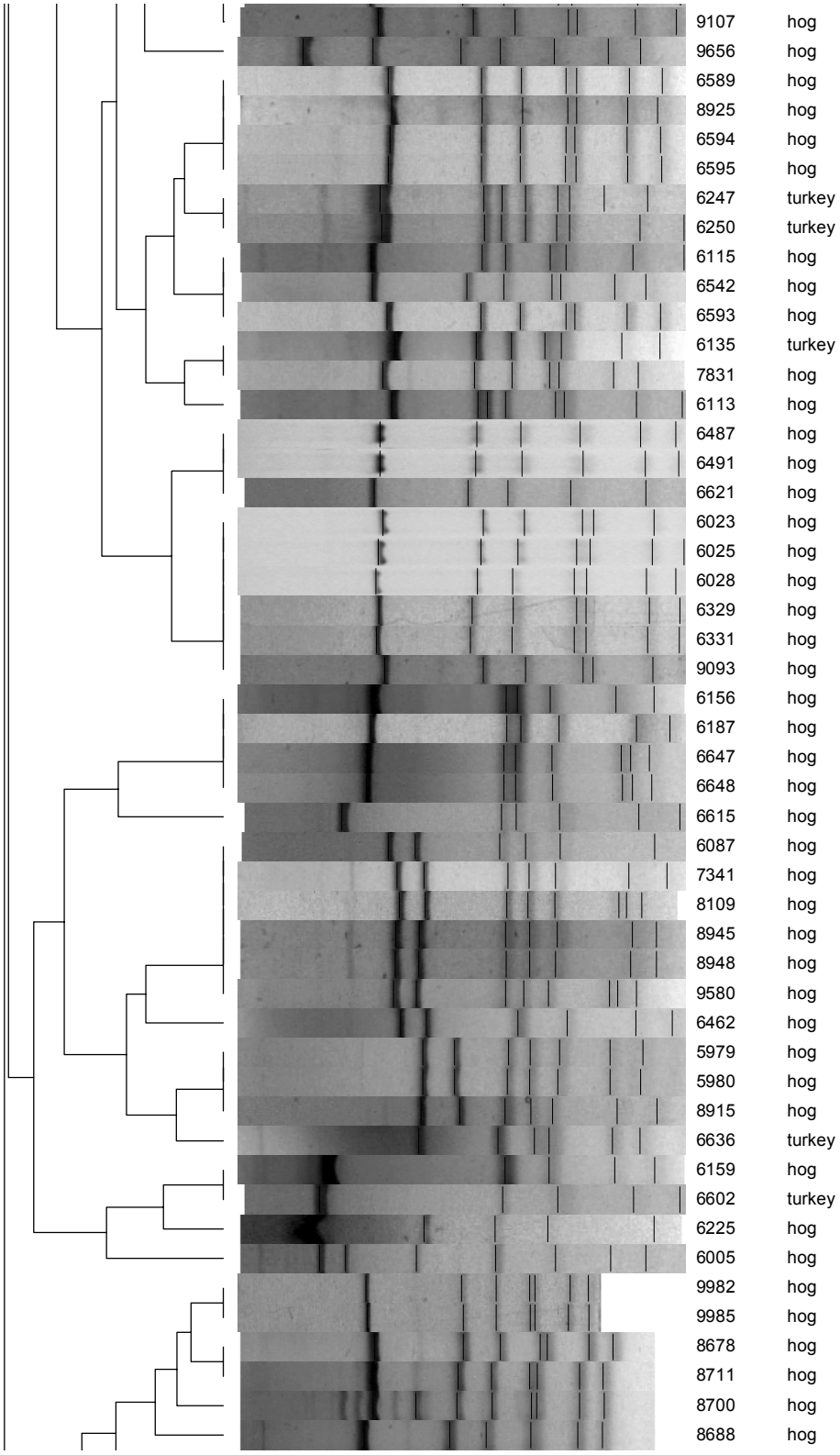

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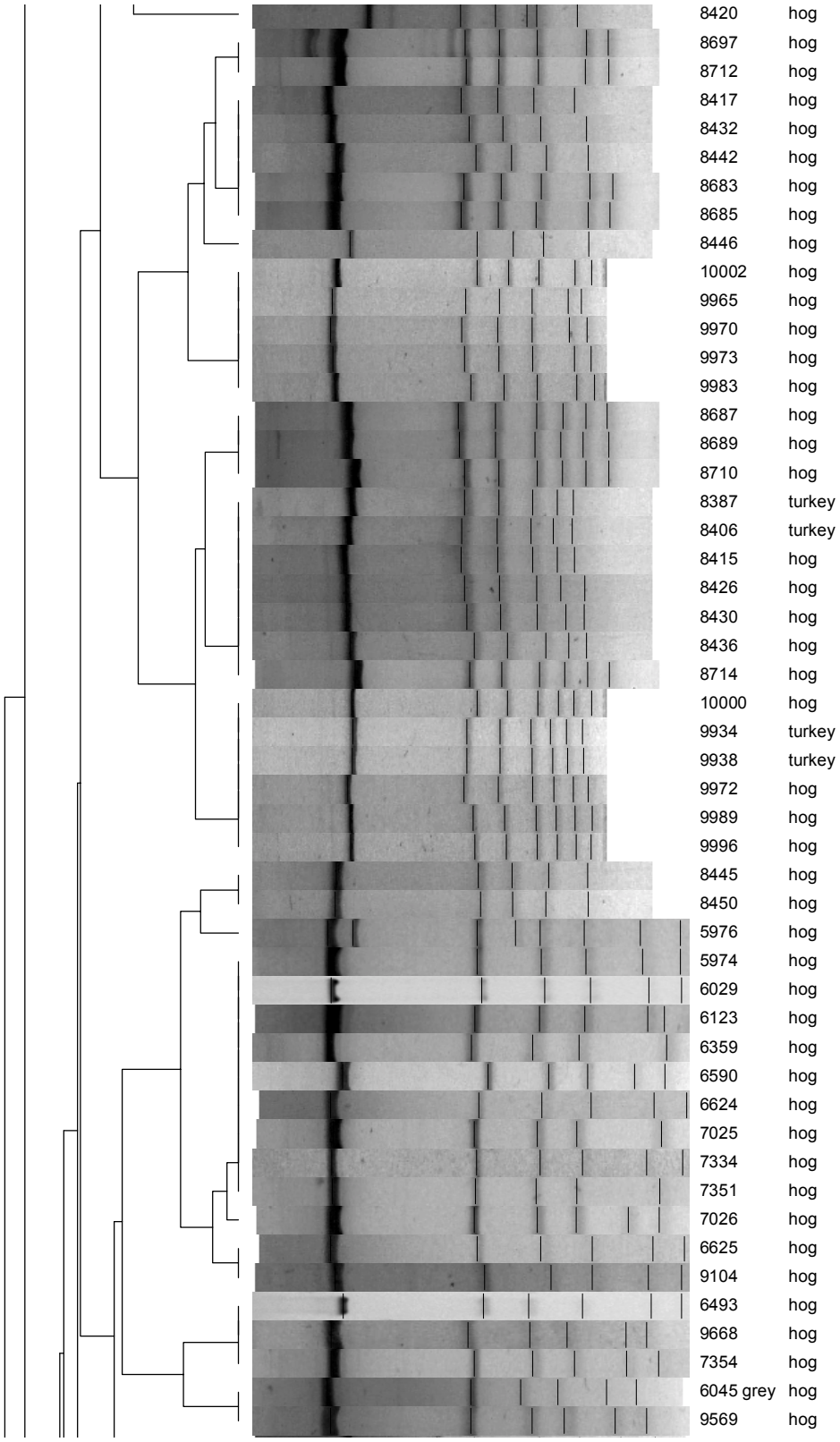

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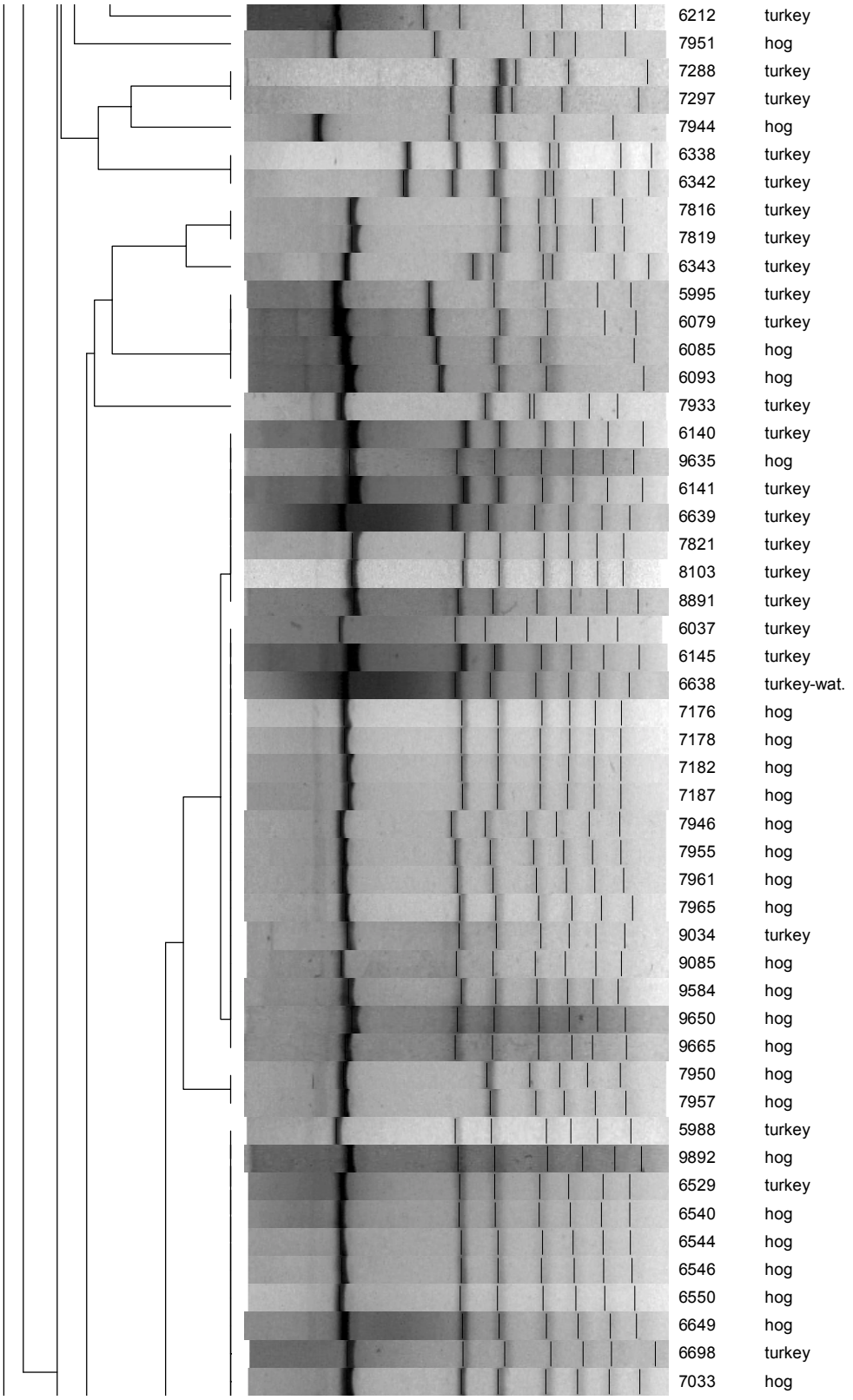

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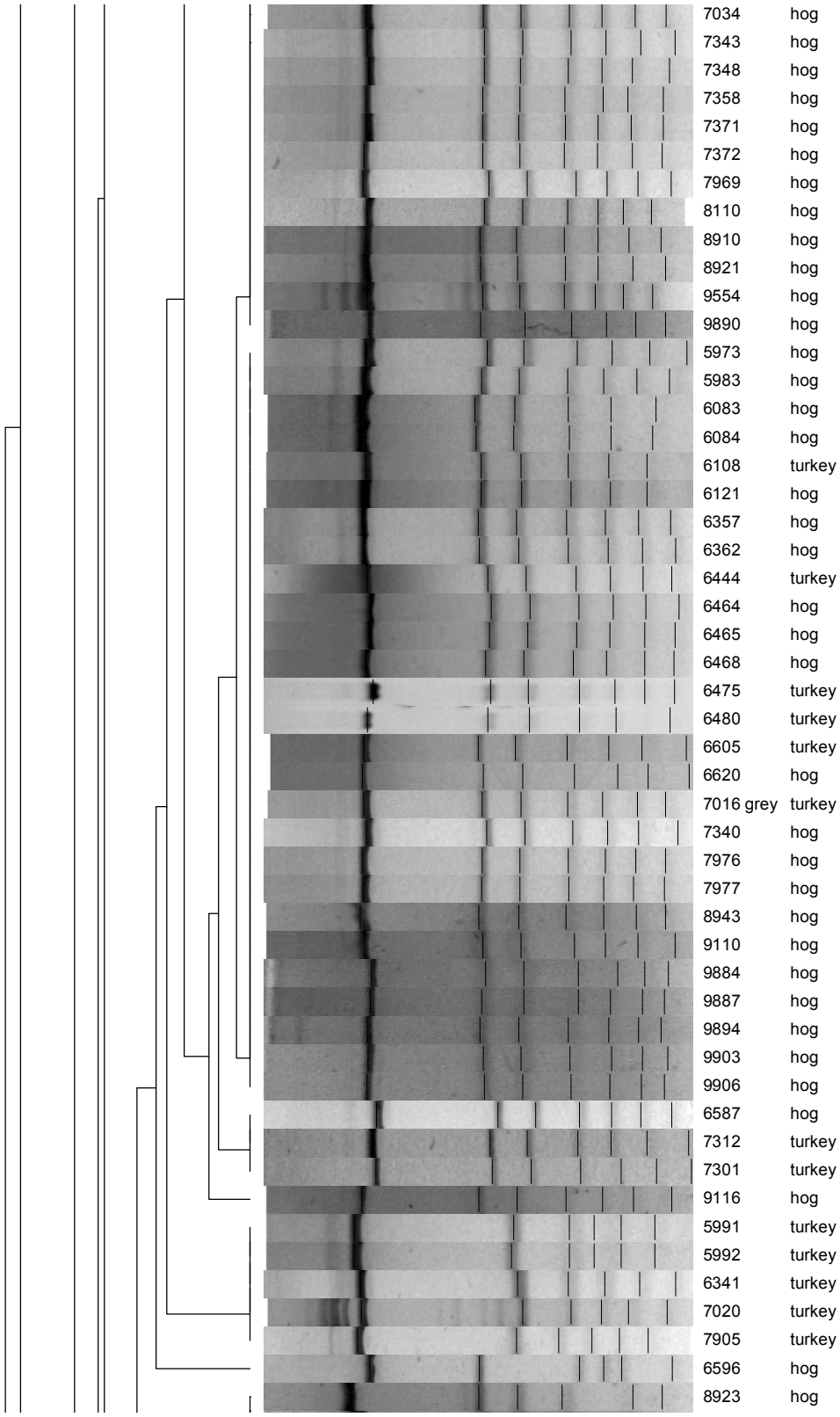

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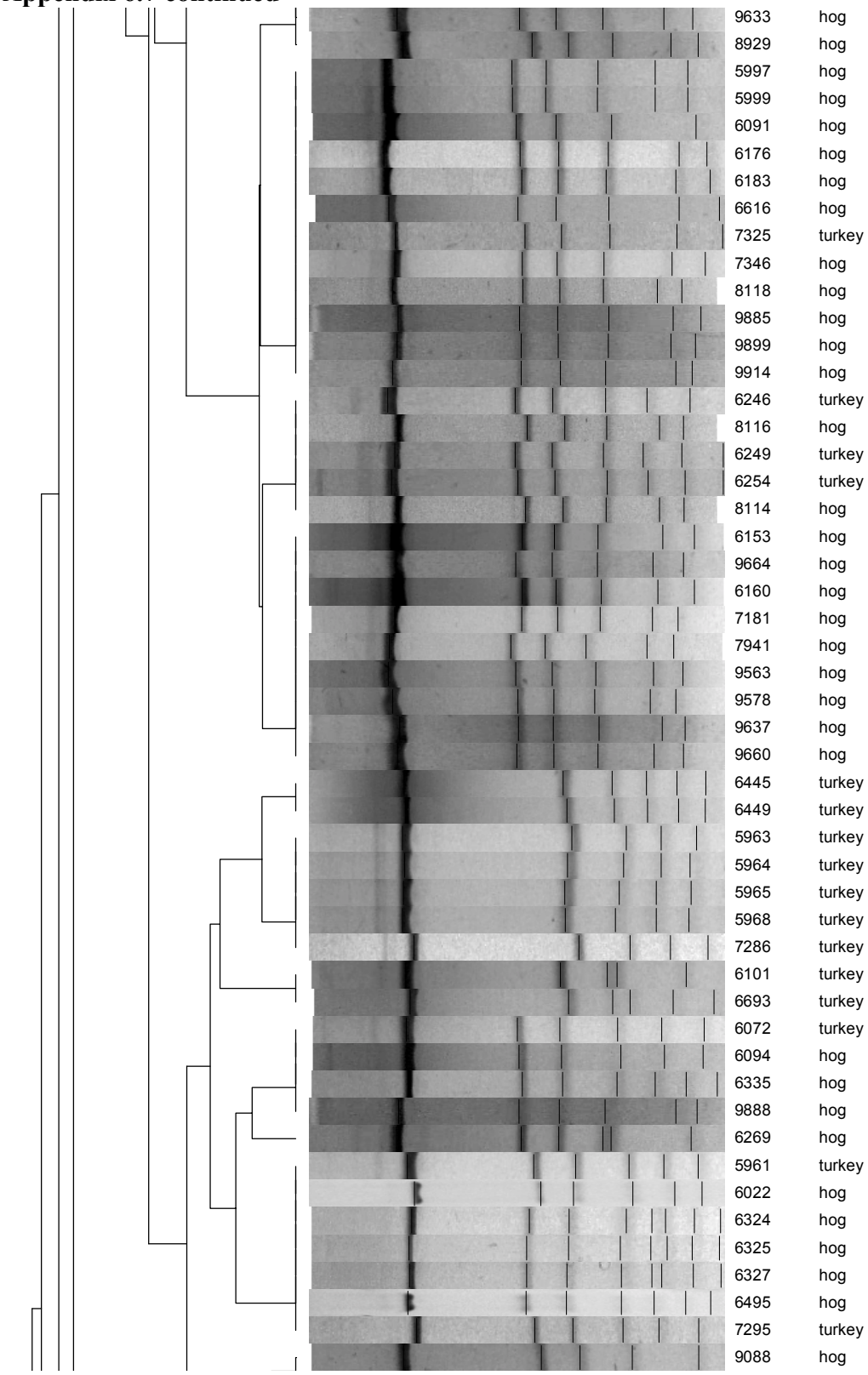

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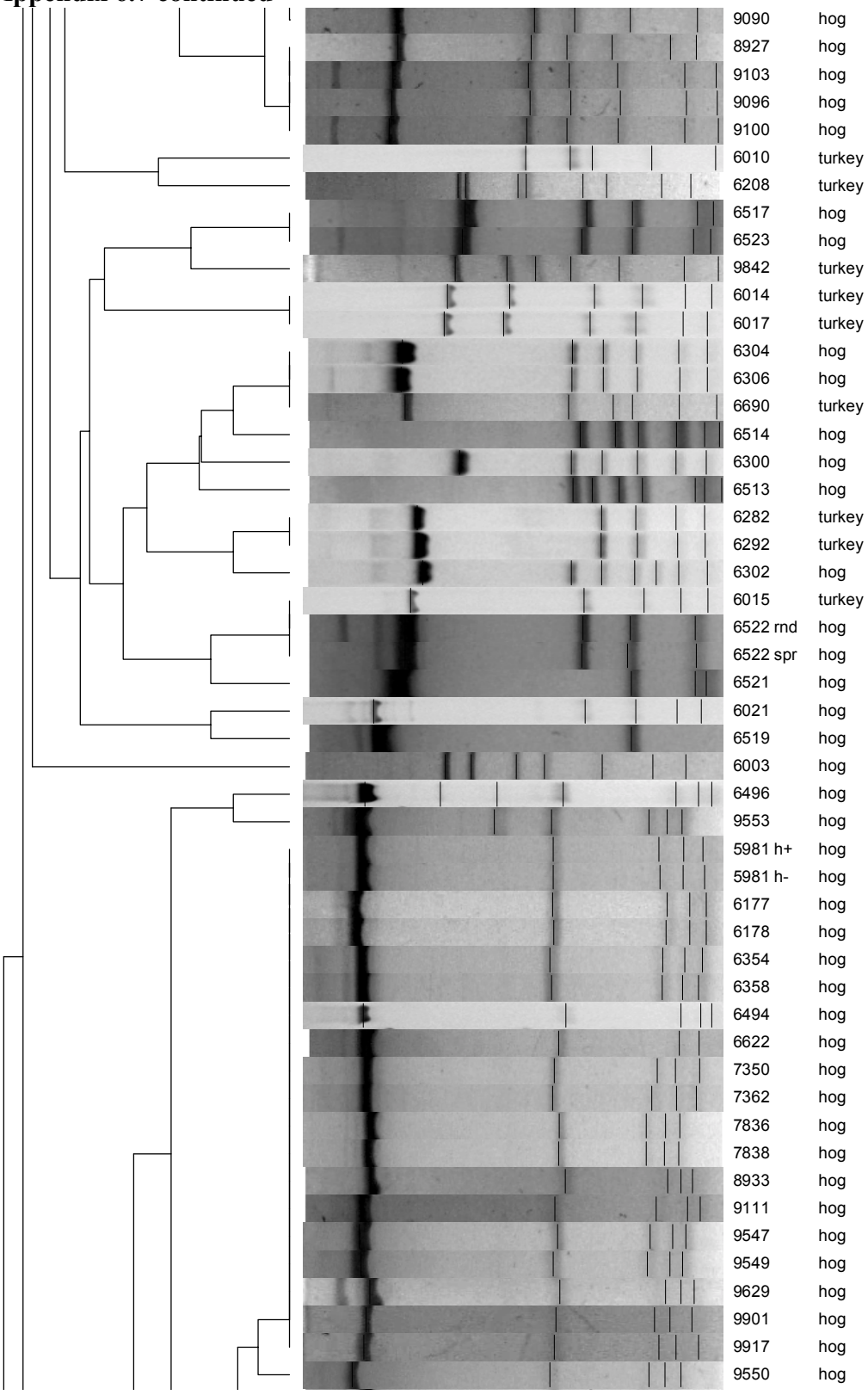

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