LONGITUDINAL STUDIES OF *ESCHERICHIA COLI*, *CAMPYLOBACTER JEJUNI*, AND *SALMONELLA* SSP. IN BROILER CHICKENS USING AUTOMATED RIBOTYPING

Except where reference is made to the work of others, the work described in this dissertation is my own or was done in collaboration with my advisory committee. This dissertation does not include proprietary or classified information.

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LONGITUDINAL STUDIES OF *ESCHERICHIA COLI*, *CAMPYLOBACTER JEJUNI*, AND *SALMONELLA* SSP. IN BROILER CHICKENS USING AUTOMATED RIBOTYPING

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DISSERTATION ABSTRACT

LONGITUDINAL STUDIES OF *ESCHERICHIA COLI, CAMPYLOBACTER JEJUNI,* AND *SALMONELLA* SSP. IN BROILER CHICKENS USING AUTOMATED RIBOTYPING

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The prevalence, distribution and characteristics of *Escherichia coli, Campylobacter jejuni,* and *Salmonella* spp. through an integrated broiler chicken operation from the day of hatch through processing were examined. Two farms were sampled during four consecutive flocks to determine the prevalence and persistence of bacteria of food safety concern in the poultry house environment. Bird movement from

the farms through the processing plant was monitored using transport crate drag swabs and carcass rinses.

An alternative method of litter sampling was employed using surgical shoe covers. In lieu of drag swabs, surgical shoe covers were a practical method of litter sampling to determine the level of flock colonization, were more effective at recovering *Salmonella* isolates, and yielded three times as many *Salmonella* serotypes as drag swabs.

Litter samples are a method of assessing flock health prior to processing. The depth at which the litter is sampled was tested to determine if isolates recovered from surface sampling methods accurately reflected the *E. coli* population in the house. Deep litter, shallow litter, and surgical shoe cover samples were compared using automated ribotyping to elucidate trends in recovery. *E. coli* isolates were recovered from all three sample types and examined to determine the genetic diversity of the isolates. There was no significant correlation among strains between flock age, house, flock number, or farm and ribogroups. Based upon the patterns of *E. coli* recovery between the different sample types, our results indicate that any of the methods are capable of recovering the common isolates although surgical shoe covers were simplest to use.

C. jejuni and *Salmonella* spp. isolates were recovered from multiple chicken house environmental samples taken on chick delivery day, mid-growout, and processing day. Birds were traced through the processing plant to determine if isolates from the farm, at any age, persisted on carcasses to the final product. The majority of *Salmonella* isolates were ribotyped as *S.* Kentucky, all of which were of same strain. All environmental samples taken on chick placement day in the first flocks were *S.* Kentucky-free but eggshells and chick mortality were positive. This indicated that the

source may have been the hatchery or breeder flocks. All *Campylobacter* isolates were ribotyped as *C. jejuni* although no isolates were recovered on chick placement day. Three core isolates were identified and persisted from the farm to the processing plant.

All in all, this study was able to create a library of ribotypes for multiple bacterial organisms and track the subtypes over the course of multiple flocks. Surgical shoe covers were found to be a suitable replacement for drag swabs and litter samples for the assessment of *Salmonella* colonization. Additionally, the *E. coli* results indicated that surface sampling methods, i.e., such as surgical shoe covers, do adequately gather a representative sample of isolates from the litter as a whole. A clonal population of *Salmonella* Kentucky indicated that the breeder flocks or hatchery was the source of the organism. *Campylobacter* samples provided information on the most effective samples for gathering multiple isolates as well as persistent core isolates that remained in the environment during multiple flocks. This database may be used in the future for longitudinal studies and comparisons.

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I. INTRODUCTION

Two bacteria, *Salmonella* and *Campylobacter*, are responsible for the majority of human food-borne gastroenteritis linked to poultry products. *Escherichia coli*, on the other hand, a bacterium that is extremely prevalent in animal production environments, causes a primary or secondary colibacillosis in chickens, as well as food-borne infection in humans. *Salmonella* has long been recognized by the public and by officials as a leading food-borne pathogen (Jones *et al.*, 1991), but recently *Campylobacter* is a more problematic foodborne pathogen. *Campylobacter* is a microaerophillic bacterium that is difficult to culture and therefore has only recently been linked to food-borne illness. Poultry is a primary reservoir for *Campylobacter jejuni* and during the 1990's *Campylobacter* was responsible for more episodes of human illness from poultry products than *Salmonella* (Mead *et al.*, 1999). The recent characterization, culture, and study of this organism is due to special growth requirements, and niches in the poultry production system that are still being investigated.

Paratyphoid *Salmonella* infections, which includes those in serogroups other than group D, do not cause the severe clinical disease in chickens and turkeys, as seen with host-adapted *Salmonella* Gallinarum and *S.* Pullorum, but are nevertheless of concern to the human consumer. The organisms often responsible for causing food-borne salmonellosis in humans are *S.* Typhimurium or *S.* Enteritidis. Annual FoodNet data (CDC, 2005) showed that, in 2004, salmonellae surpassed *Campylobacter* in the

frequency of food-borne outbreaks of human gastroenteritis in the United States. Salmonellae have been and will continue to be studied with regard to sources in the poultry house, and processing plant environments, flock illness, and human food-borne illness outbreaks.

Escherichia coli has a long history of infecting poultry, but is also capable of causing food-borne infection when present in sufficient numbers. Several severely pathogenic strains of *E. coli,* i.e., *E. coli* O157:H7, have been linked to food sources and also have a low infectious dose (Salyers and Whitt, 2002). This is of particular concern to the elderly, young, and immunocompromised persons but poultry are not a primary source of the *E. coli* O157:H7 organism (Doyle *et al.,* 1997). *E. coli* may cause illness if cross-contamination or mishandling of the product occurs in the home.

II. LITERATURE REVIEW

The focus of this review is on the dissemination of pathogenic bacteria in poultry production and the use of ribotyping as a tool to track the journey of these bacteria within the commercial broiler chicken production system. *Escherichia coli, Salmonella,* and *Campylobacter* were the three pathogenic bacteria surveyed in this study using conventional culture methodology. Each bacterial species survives differently in the poultry production continuum and therefore must be discussed individually. Molecular typing of isolates was achieved using automated ribotyping, the value and functionality of which is detailed in the following review.

Escherichia coli

E. coli are ubiquitous bacteria that were first identified and reported by Theodor Escherich in 1885. The bacterium is a Gram negative, non-sporeforming, facultative anaerobe. The rod-shaped bacterium is from 1.1-1.5 µm wide by 2.0-6.0 µm long and may or may not be motile via peritrichous flagella. Normally found in the lower gastrointestinal tract of warm blooded mammals, *E. coli* is particularly useful as an indicator of fecal contamination of foods and water (Holt *et al.,* 1994). The majority of *E. coli* strains are innocuous although some strains produce enterotoxin or other virulence factors. These virulence factors, as well as serotyping, are used to characterize pathogenic strains.

Serotyping relies upon surface antigens of the bacteria for agglutination with antibodies. The serotyping strain classification system uses the O antigen of the lipopolysaccharides (O) in the cell membrane to identify the serogroup and the flagella (H) to identify the serotype, which is more specific. Currently, there are approximately 160 serogroups. The recent emergence of virulent serotypes is cause for concern because of their association with food, low dose, and the severity of illness. Virotypes are, by definition, a typing system based upon patterns of bacterial attachment to the host cell, the effect of the attachment on the host cell, toxin production, and invasiveness. Virotypes typically contain more than one serogroup and serotype. Sharp distinctions for grouping *E. coli* strains based on virulence factors can become difficult to make as new information about some strains becomes available (Salyers and Whitt, 2002).

The six *E. coli* virotypes are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC), and diffusely adhering *E. coli* (DAEC). Localized adherence is characteristic of ETEC, EHEC, and EPEC strains, aggregative adherence is characteristic of EAggEC, and diffuse adherence is characteristic of DAEC. The variety of *E. coli* virotypes appears to be due to the carriage of virulence genes on mobile elements acquired from other bacteria (Salyers and Whitt, 2002). Food microbiologists have focused research on ETEC, EPEC, EIEC, and EHEC strains because of their clear association with outbreaks via food and water (Salyers and Whitt, 2002) and will be discussed in this review.

Commonly referred to as traveler's diarrhea, ETEC produces two forms of toxin. Heat-labile toxin (LT) and heat-stabile toxin (ST) can be produced independently of one

another or simultaneously. The heat-labile toxin is produced in two forms, LT-I and LT-II; the amino acid sequence of LT-I is 75% homologous to cholera toxin. ETEC's mode of action is to adhere to the intestinal mucosa and alter normal cellular function, causing an increase in chloride secretion into the intestinal lumen and prevention of NaCl uptake. The ion imbalance causes intestinal cells to efflux water into the lumen resulting in watery diarrhea (Salyers and Whitt, 2002).

EPEC and EIEC strains are often found in young children with persistent diarrhea. EPEC strains alter the ultrastructure of the host mucosal cells. The process of attaching and effacing the host cells is invasive and causes pronounced inflammation. Diarrhea may be caused by the process of invasion of the cell as well as by the sabotage of signal transduction systems that control ion flow (Salyers and Whitt, 2002). EIEC strains may not cause hemorrhage and result in bloody, voluminous diarrhea. The mode of action is to enter an intestinal mucosa cell and multiply most often in the colon. The organism then invades adjacent epithelial cells (Jay, 2000).

EHEC strains have recently been isolated from several food-borne outbreaks. *E. coli* O157:H7 is the prototype strain for EHEC and the cause for concern lies in its aberrant behavior, low infective dose, and the large susceptible population of people. *E. coli* O157:H7 is a typical of *E. coli* in biochemical tests and as few as 10 organisms are sufficient to cause disease. The susceptible population, includes young children, the elderly, and those who are immunocompromised. *Stx* toxin is produced by EHEC strains, which is virtually identical to Shiga toxin, the toxin responsible for dysentery. Outbreaks of the EHEC disease involving children are of concern because of the potential for sequelae such as hemolytic uremic syndrome (HUS) that results from the production of

the *Stx* toxin. The risk of HUS is reduced in older children and healthy adults. Receptors for the *Stx* toxin are found on kidney and intestinal cells, and infection of the kidneys can lead to renal failure. *Stx*2 is found more often than *Stx*1 in HUS-causing strains (Salyers and Whitt, 2002).

E. coli is found in the intestinal tract of poultry at concentrations of up to 10^6/g (Barnes *et al*., 2003). Young chickens, those that have yet to establish a normal microflora, will carry higher numbers of the organism (Leitner and Heller, 1992). Typically, 10-15% of intestinal coliforms may potentially be pathogenic (Harry and Hemsley, 1965). The current method of poultry processing may cause cross-contamination of carcasses if intestinal breakage should occur during evisceration (Berrang *et al.*, 2004; Mead and Scott, 1997).

Diseases such as colibacillosis and infectious process (cellulitis) can be a problem for poultry producers. Colibacillosis can be a localized or systemic infection caused by avian pathogenic *E. coli* (APEC) through primary or secondary infection (Barnes *et al*., 2003). Most APEC are not of concern to humans but are pathogenic for poultry (Caya *et al*., 1999). Molecular typing has indicated that relatively few clonal types are responsible for the multiple forms of colibacillosis (White *et al.*, 1993) and the toxins in APEC are less toxigenic than mammalian pathogenic *E. coli* (Blanco *et al.*, 1997).

On the farm, chickens are subjected to *E. coli* infections from multiple potential reservoirs. Although there are no known methods for reducing the level of pathogenic *E. coli* in the intestinal tract and feces of chickens (Barnes *et al*., 2003), competitive exclusion products have been successful in seeding the gut and inhibiting pathogenic strains (Hakkinen and Schneitz, 1996; Weinak *et al.*, 1981). The organism may be

transmitted through the egg and can be responsible for high chick mortality (Barnes *et al*., 2003). It is normal to recover *E. coli* in low numbers from yolk sacs because between 0.5% and 6% of eggs from healthy hens contain the organism (Harry, 1957). Along with obvious sources such as fecal matter and litter, dust has been reported to contain 10^5-10^6 *E. coli*/g (Harry, 1964). Additionally, rodent feces (Barnes *et al*., 2003) and contaminated well water can contain pathogenic coliforms (Nagi and Raggi, 1972). Chlorination of water, in conjunction with the use of closed (nipple) watering systems has contributed to a reduction in the number of colibacillosis occurrences (Boado *et al*., 1988). The pelleting process in feed production reduces pathogenic coliforms that may be present (Barnes *et al*., 2003). Wild birds are of particular concern to producers because they can carry coliforms that are adapted to avian species (Morishita *et al.,* 1999). Darkling beetles, both larvae and adults, when exposed to coliforms, can carry the organisms biologically and mechanically for up to 12 days. The beetles can also shed coliforms in their feces for 6-10 days. Chicks are more likely to become infected by coliforms via consumption of larvae but adult beetles are also capable of causing infection (McAllister *et al.,* 1996). Proper ventilation in the house will prevent ammonia damage to the respiratory tract. Chickens stressed by long term exposure to high levels of ammonia are subject to bacterial invasion (Barnes *et al.,* 2003).

A certain level of *E. coli* is to be expected on food products as food of animal origin is not sterile. Food products that have caused outbreaks of *E. coli* illness in the past include beef, milk, poultry, seafood, vegetables dishes, and water. Poultry carcasses are rinsed and tested to ensure that product does not leave the plant carrying an excess of *E. coli* organisms. Should *E. coli* O157:H7 be detected, product is subject to disposal. In

the processing plant, colibacillosis is the most frequently reported disease in surveys of condemnations (Barnes *et al.*, 2003). Although it is possible for chickens to become infected with *E. coli* O157:H7 (Doyle *et al.*, 1997), there is a low occurrence of natural infection (Barnes *et al.*, 2003). Of greater concern is the level of contamination on the final product leaving the processing plant.

Salmonella

Salmonellae are small, motile, gram-negative, non-spore-forming rods, usually measuring 0.4-0.6 x 1-3 μm in size. Optimal growth temperature for this facultative anaerobe is 37^{o}C. Colonies are usually 1-2 mm in diameter, round and slightly raised with smooth edges. Exposure to heat (60 ^{o}C) for 5 minutes is sufficient to kill 3x10^{8} *S.* Tyhpimurium cells per gram of ground chicken. Refrigeration or freezing temperatures can prolong the survivability of salmonellae on poultry carcasses (Nagaraja *et al.*, 1991).

Not all salmonellae cause the same disease. Certain *Salmonella* serotypes cause illness in poultry (host-adapted) while others are well-known as human food-borne pathogens (paratyphoid infections). The host-adapted serotypes, *S.* Pullorum and *S.* Gallinarum, cause pullorum disease and fowl typhoid, respectively. These two serotypes colonize poultry of many species and cause disease that is economically significant to the poultry industry. *S.* Pullorum and *S.* Gallinarum typically colonize susceptible chicks during the first two weeks of life and can cause an increase in morbidity and mortality (Shivaprasad, 2003). As the chickens age, their susceptibility to colonization decreases (Severens *et al.*, 1944). In contrast, paratyphoid serotypes can colonize chickens of any age and do not always cause disease (Gast, 1997). Paratyphoid *Salmonella* serotypes are

motile (Gast, 2003) while *S.* Gallinarum and *S.* Pullorum are non-motile (Shivaprasad, 2003). It is as the agent of foodborne infection in humans that paratyphoid serotypes have been studied intensively. There are over 230 different paratyphoid serotypes found in poultry, although a far greater number of serotypes and hosts exist (Gast, 1997). This review will emphasize the characteristics of paratyphoid *Salmonella* serotypes in poultry production.

It has been suggested that 75% of chicken and turkey flocks in the United States have been infected with one or more *Salmonella* serotypes at some stage of their life (Snoeyenbos, 1977). Economic losses to the United States poultry industry in the 1970's due to paratyphoid infections were estimated at $77 million annually (Marsh, 1976). With these losses in mind, the industry has cooperated with researchers over the years to determine the sources of infection. For salmonellae, there are multiple potential sources of infection for a flock. A broiler, or carcass, risks colonization and contamination via biosecurity or sanitation failures.

Chicks can be colonized by salmonellae during the first two weeks post-hatch. Peak mortality in naturally infected broiler flocks occurs at 4 days of age (Morris *et al.*, 1969). The acquisition of normal flora is often the reason stated as to the ability of young poultry to avoid colonization by pathogenic bacteria (Nagaraja *et al*., 1991). As chicks age, increasingly large doses of *S.* Typhimurium are needed to produce a detectable infection. Milner and Shaffer (1952) found that in the 24 hours post-inoculation, 95% of 1-2 day old chicks dosed with 1000 *S.* Typhimurium were positive, 51% of chicks dosed with 10 *S.* Typhimurium were positive, and 5% of chicks dosed with 1-5 *S.* Typhimurium

were positive. At four days post hatch, larger doses were required and only irregular infection was produced.

Fecal contamination of the egg surface can occur (Board, 1966) and salmonellae can be transmitted to offspring through the egg. No matter how clean and prepared a producer's house, the arrival of infected chicks can quickly nullify those efforts. Infected parent flocks can increase the risk of *S.* Typhimurium in progeny flocks markedly, and antibiotic treatment of chicks may not be effective because horizontal transmission within the flock can occur before the chicks arrive at the farm (Chriel *et al.*, 1999).

The hatchery is a point of horizontal transmission for salmonellae. Fluff, aerosol, and infected eggs that explode all have the potential to spread salmonellae to all chicks in the hatcher. *Salmonella* is able to survive in hatchery fluff samples stored at room temperature for up to five years (Miura *et al*., 1964). If not cleaned effectively, hatchers can remain positive and infect subsequent hatches for several weeks or months (Nagaraja *et al.*, 1991). Given this situation, chicks may become infected before they leave the hatching cabinet. Pooling eggs from several flocks in a hatcher is a common practice but doing so may cause an uninfected parent flock to appear positive (Chriel *et al.*, 1999).

Poultry feed is an important source of *Salmonella* infections. Jones *et al.* (1991) found that 60% of meat and bone meal samples from feed mills were contaminated and *Salmonella* was subsequently isolated from 35% of mash feed samples. Non-pelleted feeds are more frequently contaminated than pelleted feeds (Morris *et al.*, 1969), and *Salmonella* isolation rates can be reduced by 82% during the pelleting process (Jones *et al.*, 1991).

Horizontal transmission of several organisms, including *Salmonella*, within the poultry house can also occur through the water. As early as two weeks after chick placement, salmonellae isolations from water troughs were more frequent than from litter (Morgan-Jones, 1980). Nakamura *et al.* (1994) showed that uninoculated hens that shared the same drinking water as *S.* Enteritidis inoculated hens excreted the same quantities of organisms just one day post-inoculation. Open drinkers may be the reservoir with the highest incidence of *Salmonella* contamination toward the end of the growing period (Lahellec *et al.*, 1986). If *Salmonella*-positive feed is given to poultry, then open water containers may become colonized through contamination with feces or by chickens carrying the food on their beaks (Nagaraja *et al.*, 1991).

Rodents are a major reservoir for salmonellae given their ability to easily access a poultry house (Nagaraja *et al.*, 1991). During one 24-hour period, one mouse can excrete an average of 100 fecal pellets and each pellet may carry $2.3x10^5$ *S.* Enteritidis organisms. *S.* Enteritidis can persist in a mouse population for at least 10 months (Henzler and Opitz, 1992). Three-week-old chicks can become infected orally through feed and water contaminated with mouse droppings (Davies *et al.*, 1995a). Rodents acquire and move paratyphoid *Salmonella* easily and can quickly recontaminate the environment of depopulated and cleaned poultry houses. Failure to eliminate an infected mouse population was deemed the most important hazard for subsequent flocks (Davies and Wray, 1996b).

Insects are also a likely source of *Salmonella* within the poultry house. They are both mechanical and biological carriers of salmonellae. German, Oriental, and American cockroaches can become infected with salmonellae and pass it on to other cockroaches as

well as contaminate equipment (Kopanic *et al*., 1993; Kopanic *et al*., 1994). Lesser mealworms can carry salmonellae. The beetle feeds on dead and dying chicks, feces, feed, and organic matter in litter. Larvae dosed with $3x10^8$ *Salmonella*/ml excreted bacteria in their feces for 28 days. The larvae can carry the organism through the pupal stage and into the adult stage. Most chicks, if they consume adults or larvae that are infected, will shed salmonellae within 24 hours (McAllister *et al.,* 1994).

Equipment is an important vector of *Salmonella*. Proper cleaning and disinfection of equipment prior to restocking the house is key to prevention of hazards for the next flock (Davies *et al.*, 1995b). Foot traffic to and from contaminated vehicles is also considered a risk and appropriate biosecurity measures are required to prevent introduction of *Salmonella* from human footwear (Davies *et al.,* 1997).

The litter and walls are two areas that can harbor organisms that escape cleanout; potentially infecting newly arrived chicks. Lahellec *et al.* (1986) found *Salmonella* on the walls within one day of chick placement, making the walls the most important source of contamination for the flock. Used litter is often utilized in poultry facilities, thus reducing the operational costs of producers. Used litter helps the chick to establish a natural intestinal flora but at the risk of infection by pathogens such as *Salmonella*. Litter treatment products may be used on the litter to alter the pH, thus making the environment inhospitable to *Salmonella* (Nagaraja *et al.*, 1991).

Crating, transport, and processing can lead to an increase in the load of bacteria on the carcass (Carraminana *et al.*, 1997; Rigby and Pettit, 1995; Stern *et al*., 1995). There is interest in processing *Salmonella*-negative flocks prior to positive flocks. Permitting “clean” flocks to be processed prior to “dirty” or infected flocks could aid in

decreasing the levels of cross-contamination in the plant although not all researchers believe this will produce the desired effect (Nauta *et al*., 2005).

The most significant points of cross-contamination within the poultry processing plant are the scald tank, feather picking machine, and chill tank (Wempe *et al*., 1983). At these locations carcasses are either placed in an environment where there is infrequent cleaning during the processing day or, in the case of the chill tank and scald tank, water may be recycled for several hundred carcasses, and there is constant mixing. Inside the processing plant the feather picking equipment can be a source of cross-contamination between or within flocks. Rubber fingers within the feather picking machine are a point of contact for every carcass that passes through. *Salmonella* can be passed from the skin of one carcass to another (Wempe *et al*., 1983).

Both the scald tank and chill tank permit *Salmonella* cross-contamination through the water. If not maintained adequately, both systems can increase the load of bacteria on the carcass. Humphrey and Lanning (1987) adjusted the scald tank water pH by the addition of alkaline products until a pH of 9.0 was reached. There was no effect on the number of carcasses positive for *Salmonella*. To prevent the co-mingling of carcasses that occurs in chill tanks, some companies have switched to a forced-air chilling method. Each method has its drawbacks, but those who use chill tanks often chlorinate the water to reduce the microbial load, although alternate sanitizing agents in chill tanks have been investigated (Dickens and Whittemore, 1995; Thiessen *et al*., 1984; Russell and Axtell, 2005).

Salmonella is not easily removed from carcasses. The rate of *Salmonella* contamination of chicken carcasses and parts from Ohio retail markets was reported to be

43% (Bokanyi *et al*., 1990). False-negatives may be declared if a single carcass rinse fails to dislodge *Salmonella* that is firmly attached to the skin. One study found that the organism may not be recovered on the first rinse, but rather on the third, fifth or tenth rinse in carcass rinses performed 5-10 consecutive times (Lillard, 1989).

Campylobacter

Campylobacter is a small, motile, Gram-negative, non-spore-forming, spirally, curved rod. It usually measures 0.2-0.8 μm in width and 0.5-5 μm in length. Optimum growth temperature for this microaerophile is 42^{o}C. Microaerophiles prefer very little oxygen (5%), 10% carbon dioxide, and the balance of atmosphere (85%) consisting of nitrogen (Shane, 1991a). Colonies are also identified as *Campylobacter* by using tests such as catalase (+), oxidase (+), and Gram staining (Nachamkin, 1999).

It is thought that 90% of broilers, 100% of turkeys, and 88% of domestic ducks carry *Campylobacter* (Shane, 1991b). In the past, economic losses due to *Campylobacter* infection of humans each year exceed those of *Salmonella* based on the higher number of infections (2.2-2.4 million annually) in the United States (Saleha *et al.*, 1998). Flock testing, or carcass testing in the processing plant, has not been mandated. Producers and processors, in anticipation of future regulations, have worked with researchers to determine sources of infection on the farm but progress has been slow. Continued research will be necessary until our knowledge of this organism meets or exceeds what is known about other food-borne pathogens such as *Salmonella*.

C. jejuni is rarely found in day-old chicks but is quite often found in chickens beginning at about the second week (Shane, 1991a). Although chicks do not necessarily

arrive at the farm with *C. jejuni*, the organism will spread rapidly through the flock after approximately two weeks of age. Once detected, the majority of the flock will become colonized in one week's time (Lindblom *et al*., 1986; Neill *et al*., 1984). A minimum dosage for cloacal challenge at $<10^2$ colony forming units (cfu) and 10^4 cfu for oral challenge resulted in 88% positive chicks after three days and 97% positive by after seven days post-infection (Shanker *et al*., 1988). Within the chicken intestinal tract, *C. jejuni* prefer locations in the lower gastrointestinal tract, especially the ceca. This anaerobic environment is hospitable to *C. jejuni* where they colonize the crypts of the villi (Saleha *et al.*, 1998). Colonization occurs in numbers anywhere between 10^4-10^7 cells per gram of cecal material (Beery *et al.*, 1988).

Water can serve as a reservoir for *Campylobacter* infection of poultry flocks. Water containing 10^2-10^9 cfu/ml of *C. jejuni* colonized nearly all chicks in isolators within a week (Shanker *et al.*, 1990). *C. jejuni* can and will survive in water, but it may not always be culturable. During the first four days of exposure to an aquatic environment, *C. jejuni* develops a high injury level due to sublethal stressors that are inherent in aquatic environments (Terzieva and McFeters, 1991). Given the susceptibility of the organism in this damaged state, maintenance, as well as the cleaning and disinfection of the watering system, should be effective in eliminating the bacteria. The thorough cleaning of the water distribution system in houses may reduce or eliminate an outbreak strain of *Campylobacter* that may be found on broiler farms (Pearson *et al.*, 1996).

Rats can transmit *Campylobacter* in broiler operations. One farm reported an 87% prevalence of *C. jejuni* in rat ceca and rodent dropping in feeders may be consumed

by chickens (Shane, 1991a). After infection, mice can carry and shed *C. jejuni* for at least six months. One day post-exposure, a mouse can shed $\log_{10}$ 3.48 to $\log_{10}$ 5.21 cfu/g of feces. After six months, mice may shed the organism at levels of $\log_{10}$ 2.70 to $\log_{10}$ 6.15 cfu/g of feces. This indicates that a rodent population that remains in a poultry house after cleanout may inoculate subsequent flocks (Berndtson *et al*., 1994).

Wild birds are carriers of *C. jejuni* and the organism is a normal component of the intestinal flora of several bird species. Bird species that have been caught in or around poultry houses include pigeons, swallows, and sparrows, all of which carried the organism. Should birds gain entry to broiler houses, they have the potential to transmit *C. jejuni* and other enteropathogens. Craven *et al.* (2000b) found *C. jejuni* and other bacterial pathogens in a study of dry/wet droppings, intestinal contents, and cloacal swabs of wild birds at a poultry farm.

Houseflies and beetles are implicated as biological and mechanical carriers of *Campylobacter*. Fifty percent of captured flies from inside or outside poultry houses in Norway were positive for *Campylobacter*. Another controlled study noted that flies, either through regurgitation or defecation, were the sole source of infection. This study could not rule out mechanical transmission because 10% of the fly feet examined were positive for *Campylobacter* (Shane, 1991a). Litter beetles can infect chickens through the ingestion of larvae or adults. A study by van de Giessen *et al.* (1998), found that insects and broilers on a farm had identical biotypes of *Campylobacter*, indicating that insects may have been a vehicle of transmission and could perhaps infect future flocks.

Several experiments have indicated that litter in poultry houses is an excellent source of *Campylobacter*. It is common practice for producers to retain litter for use with

subsequent flocks in order to reduce operational costs. Used litter can help chicks attain a natural flora in the gut much sooner than fresh litter but the risk of the chick becoming infected with a pathogen is also increased. In one study, chicks placed on litter that was inoculated with 10^6 cfu/g *C. jejuni* began shedding the organism within five days. The broilers continued to shed the pathogen until a market age of 46 days was reached (Shane, 1991a). Chickens that are infected tend to remain infected for extended periods due to coprophagy and a continually colonized environment. Due to the inhospitable nature of litter, *C. jejuni* does not tend to remain viable for long without chickens present. Another study noted that once infected chickens were removed, the *C. jejuni* could not be isolated from environmental samples of litter, water, and feed after 72 hours (Shanker *et al*., 1990). The water activity of litter is apt to be the key to *Campylobacter*'s survivability since it does not tolerate desiccation (Shane and Stern, 2003; Luechtefeld *et al*., 1981.).

Feed does not seem to be a likely source of infection due to the low moisture content. Feed from the feed mill and feed bins have been ruled out through repeated experiments by several researchers. One study recovered *C. jejuni* from a feed trough but the trough may have been contaminated via feces, feed regurgitation, or beak transfer of the organism (Shane, 1991a).

It is widely believed that vertical transmission of *Campylobacter* within the egg does not occur but the hatchery can act as an amplifier if there are infected chicks in the hatcher (Clark and Bueschkens, 1988). Semen, which must pass through the cloaca during ejaculation, has yielded *Campylobacter* (Cox *et al*., 2002) but the testes and ductus deferens remained free of the organism (Cox *et al*., 2003). Since the egg passes

through the cloaca during oviposition, it is possible that hens can transmit the organisms to the outside of the egg (Newell and Wagenaar, 2000; Cox *et al*., 2003). This route appears unlikely due to *C. jejuni*'s sensitivity to drying and studies indicate that chicks arrive on the farm free from *Campylobacter* infection (Berndtson *et al.*, 1996a). Chicks could be a rare source of infection on the farm. Once at the farm, *C. jejuni* appears to be amplified via horizontal transmission through the flock.

Percent recovery of *C. jejuni* on poultry products varies depending upon the levels of contamination of carcasses and parts. Dirty coops allow for cross-contamination in transport (Berrang *et al*., 2003). Immersing broilers in scald tank water does not always decrease *Campylobacter* levels, especially if the water is too cool (Wempe *et al*., 1983) or has an ineffective pH (Humphrey and Lanning, 1987). The prevalence of *C. jejuni* increases during picking and evisceration but decreases somewhat during the chilling process. *Campylobacter* can survive chilling and storage at low temperatures for long periods of time (Shane, 1991a).

Automated Ribotyping

Epidemiological studies employ genetic markers to trace the dissemination of bacterial strains. Multiple molecular techniques have been employed to track the source of suspect organisms in the environment. Both phenotypic and genotypic techniques have been used in the tracing of isolates responsible for outbreaks. The discriminatory power of genotype subtyping methods are very powerful because subtypes must, as a rule, differ in the DNA they carry. Genetic differences between isolates may not encode differences in phenotypic markers such as enzymes, antigens, or antimicrobial

susceptibility. As such, the functionality of phenotypic methods in the subtyping of isolates is limited (DeCesare *et al.*, 2001).

Serotyping, as a phenotypic method, has long been employed by researchers in the pursuit of isolates responsible for outbreaks and is often used to compare the effectiveness of genotypic techniques. As it stands, this "gold standard" has its drawbacks in standardization, cost, and time. Most isolates, in order to achieve standardization, are sent to a reference laboratory. Pulsed-field gel electrophoresis (PFGE) and automated ribotyping (RiboPrinter®) are among the recently developed genotypic methods used to enhance bacterial source tracking (Manfreda and DeCesare, 2005).

Ribotyping is a southern analysis of restriction digests of bacterial DNA using probes to find fragments with information from the ribosomal RNA genes. Genetics-based ribotyping provides a method, independent of the organism, that can differentiate beyond the species level with exceptional accuracy. The automation of this process led to the creation of the RiboPrinter® Microbial Characterization System (Qualicon, Wilmington, DE, USA) which uses genetic "fingerprints" to identify and characterize microorganisms (Fritschel, 2001). The RiboPrinter® System identifies the bacterial genus and species level through the analysis of genomic fragments generated by restriction digestion of rRNA operons. rRNA operons are highly conserved regions of the bacterial chromosome found in large numbers (DeCesare *et al.*, 2001). The identification of bacterial genus and species level is performed through an analysis of an operon's most conserved regions. The discrimination between strains of a specie is

performed through analysis of the variable regions of rRNA operons and their flanking regions (Bruce, 1996).

The automation and computer-based design benefits the laboratory with a reduction in labor costs, standardization, decreased completion time, and better between-run comparisons. Between-run comparisons are useful when comparing large groups of organisms or when comparing organisms over a long period of time as seen in ongoing outbreaks (Hollis, *et al.*, 1999). The subjective interpretation of results is eliminated through built-in data analysis software. This software, in concert with web-based networking, permits the expansion of databases for comparison by researchers worldwide (Tseng *et al.*, 2001).

All reagents and material used in the RiboPrinter® come preprepared and prepackaged. The only manual portion of sample preparation is the harvesting of cells from agar plates. The RiboPrinter® consists of four modules: 1) the DNA preparation module, 2) the separation and transfer module. 3) the membrane-processing module, 4) the detection module. All automatic processes are carried out by the liquid dispensing and dilution pipette as well as the membrane-processing pipette. Prior to beginning the run, prepackaged reagents are placed into the RiboPrinter®. The prepackaged components include the DNA preparation carrier, gel cassette, nylon membrane, and membrane processing brick. There are two bays in the separation and transfer module as well as in the membrane-processing module. Therefore, sample runs can be staggered to allow up to 32 isolates to be run in a 24 hour period (Bruce, 1996; Oscar, 1998).

The function of the RiboPrinter® is outlined below in the next several paragraphs and was compiled from descriptions provided by Bruce (1996) and Oscar (1998).

Isolated bacteria are inoculated and grown overnight on brain heart infusion (BHI) agar or blood agar plates. A small group of cells is selected from the plate and transferred to a microcentrifuge tube containing buffer. The cells are resuspended via vortexing. An aliquot of this sample is transferred to a sample carrier where it is subjected to a heat treatment to reduce the viability of and inactivate nucleases. Upon completion of the heating cycle the temperature is reduced and lytic enzymes are added. DNA released by lytic enzymes is digested with the restriction endonuclease best suited for the sample. The DNA restriction fragments are then size-separated by electrophoresis on an agarose gel. The fragments are transferred to a nylon membrane and are denatured. The fragments are hybridized to a DNA probe for the ribosomal RNA operon of the bacterial species. The membrane is washed and treated with a blocking buffer and an antisulfonated DNA antibody/alkaline phosphatase conjugate. Unbound conjugate is removed through a series of washes and a chemiluminescent substrate is applied. Next, the membrane is heated and placed in front of the camera which visualizes the chemiluminescing bands. The image is captured, digitized, and sent to the computer workstation. Isolates are identified by band matching of their ribotype pattern to an existing ribotype pattern in the RiboPrinter® database.

The RiboPrinter®'s value to food safety is in its ability to track the ecology of *Salmonella*'s populations. The RiboPrinter® is a comprehensive tool that supports Hazard Analysis of Critical Control Point (HACCP) plan intervention strategies in the plant (Bruce, *et al.*, 1998). The RiboPrinter® is capable of epidemiological tracking of pathogens from the environment to the final product. Several researchers, working with different bacterial species, are attempting to find bacterial linkage among isolates

collected throughout the entire food chain by using the RiboPrinter® system (Batt, 1997; Bailey, 1998; Bruce, *et al.*, 1998; Wachtel, *et al.*, 2002).

Traditional culture and isolation methods may be employed when following isolates identified in an outbreak. Several poultry processors have successfully implemented two such methods, one for detection (Bailey, 1998) and the other for identification and tracebacks (Bailey *et al*., 2002), in the poultry processing plant. The BAX system (Qualicon, Wilmington, DE), a commercial PCR-based system, is a fast and easy approach to bacteria detection. This system is not as labor intensive as most PCR regimens and it also permits high throughput of samples. Processing plants that use the BAX system in conjunction with the RiboPrinter® system find the combination useful to detect and then locate the source of organisms. The BAX system was able to detect *Salmonella* more frequently (15%) than cultural methods (12%), when sampling chicken rinsate, with a 98% agreement in methods (Bailey, 1998). The BAX system's value to food safety is two-fold: 1) the system can detect *Salmonella* down to 1 cfu/25g of sample, and 2) the system can find *Salmonella* among high numbers of competitor organisms.

One of the oldest and most relied upon methods of bacterial identification is serotyping. Serotyping is the phenotypic method by which most new genotypic techniques are judged for accuracy. In a study by Oscar (1998), the ability of the RiboPrinter® to type *Salmonella* isolates at the serotype level was weighed against serotyping. Using EcoRI as the restriction enzyme, the RiboPrinter® was able to identify 29% of isolates to the serotype level, 9% to the strain level, 39% to the genus level, and 26% were not identified. DeCesare *et al*. (2000 and 2001) determined the optimal

restriction enzyme for automated ribotyping of *S.* Typhimurium and *S.* Enteritidis to be *PstI* or *PvuII*, but not *EcoRI*.

Serotyping is now being replaced by the faster, PCR-based methods of denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), REP, and RAPD. Albeit slightly different, pulsed-field gel electrophoresis (PFGE) is also a highly regarded method of bacterial genome identification. In one study, researchers tested the RiboPrinter®'s ability to separate outbreak strains of *Salmonella* from sporadic isolates. The RiboPrinter® method has proven to be more discriminatory than phage typing and PFGE combined (Clark *et al.*, 2003) when differentiating between sporadic isolates. Automated ribotyping, does stand apart from the other methods with its relative novelty. In one study, researchers performed comparisons of some of the methods mentioned above and concluded that combining two or more of the methods will yield the clearest and most complete information about isolates of interest (Nielsen, *et al.*, 2000).

Many genotypic techniques are based on PCR methodology. One of the drawbacks associated with these current assays is the complexity of sample handling. Another drawback is in the quantification of the initial number of bacteria. A qualitative answer may be sufficient but quantitative results may be quite valuable to HACCP plans. The problem of sample handling has been tackled in the automation of several steps of the process. The quantification challenge has also been met with the development of fluorogenic probes (TaqMan™ PCR, Perkin Elmer, Applied Biosystems Division, Foster City, CA). TaqMan™ PCR is best used for the detection and quantitation of bacteria

while the RiboPrinter® is capable of identifying bacteria and tracing reservoirs; making this combination a formidable asset to food industries (Batt, 1997).

PFGE profiles of related strains can be altered by a variety of genetic phenomena such as point mutations in restriction sites and genomic rearrangements. A study compared several *Salmonella* genotyping methods, including PFGE and RAPD, and determined that automated ribotyping, despite certain drawbacks, was equivalent to the aforementioned methods (Clark *et al.*, 2003). In a comparison of the RiboPrinter® with PFGE, 18 species of Gram negative and Gram positive bacteria were tested. A total of 411 isolates were tested with the conclusion that the RiboPrinter® was less discriminatory than PFGE. Interestingly, the RiboPrinter® was able to identify all species tested but PFGE could not type 2.75% of the isolates. The researchers in this study maintained that PFGE was still a "gold standard" method but that the RiboPrinter® had benefits through automation, standardization, and had a better between run comparison rate. A superior between run comparison rate is useful when studying outbreaks over an extended period of time (Hollis, *et al.*, 1999).

For the three genera of bacteria discussed here, optimal restriction enzyme identification is still underway. For *E. coli*, the restriction enzyme *EcoRI* accurately identifies isolates and creates ribogroups. *E. coli* O157:H7, on the other hand, has benefited from the use of *PvuII* for further characterization (Fritschel, 2001). If using more than one restriction enzyme for *E. coli* identification, the best combination is *HindIII* and *PvuII*. *HindIII* alone has been identified as an excellent enzyme for *E. coli* reservoir tracking (Tseng, *et al.*, 2001). *Salmonella* can be well characterized using both *EcoRI* (Hilton and Penn, 1998) and *PvuII* (Fritschel, 2001; Oscar, 1998), but when

working with *S. enteritidis* and *S. typhimurium*, the enzyme *PvuII* provides better discrimination (DeCesare, *et al.*, 2001). Additional enzymes, such as *PstI* with *SphI*, have been tried in combination in hopes of achieving the greatest discrimination between foodborne *Salmonella* isolates. *Campylobacter* isolates from poultry are fairly new to the automated ribotyping process but the best enzyme identified thus far has been *PstI* (Fritschel, 2001; DeBoer, *et al.*, 2000) although good differentiation was seen with *HaeIII* (Nielsen, *et al.*, 2000).

Many bacteria have been examined in hopes of finding reservoirs, whether it be on the farm or within a processing continuum. Some studies have used *Listeria monocytogenes* as a model for diagnostics because of its potential for transmission and the estimated 1400 illnesses annually (Batt, 1997). One study followed *E. coli* in cabbage that had been accidentally irrigated with creek water which was contaminated with unchlorinated tertiary-treated sewage. Using the RiboPrinter®, it was determined that isolates recovered from roots matched nonpathogenic food safety and human isolates in the RiboPrinter® database. The isolates identified by the database came from humans, domestic and wild animals, birds, or other food sources (Wachtel, *et al.*, 2002).

A thorough review of molecular typing of *Campylobacter* and *Salmonella* in poulty and poultry products indicates that more research to date has been done with *Salmonella* (Manfreda and DeCesare, 2005). Additionally, this review explains different typing methods, their value to the industry, and research on the two bacterial pathogens so far. Very little work has been done with the RiboPrinter® and *Campylobacter* in poultry and, in contrast, more research has been done with *E. coli*, especially with regard to virulence determinants (Manfreda and DeCesare, 2005). In the poultry processing

plant, the RiboPrinter® has been used to identify suspicious bacteria and their reservoirs, namely *Salmonella* and *Listeria*. *Salmonella* isolates recovered from poultry feces, carcass rinses, scald water, transport cages, paper pads, water cups, litter, cecal contents, dust, fly samples, drag swabs, post-transport cages, boot swabs, post-scald water, and water lines, yielded a RiboPrinter® identification rate of >80% when using the enzyme *PvuII* (Bailey, *et al.*, 2002). Oscar (1998) used automated ribotyping on *Salmonella* isolates collected from hatchery swabs, eggshells, litter drag swabs, feed, feed equipment swabs, flies, beetles, well water, sludge, ceca, whole broiler feather rinses, and carcass rinses. Results of this study yielded an identification rate of 38% at or below the serotype level, and 39% at the genus level, using both *EcoRI* and *PvuII,* indicating that certain restriction enzymes are not as powerful when attempting to identify *Salmonella* serotypes. Bruce, *et al.* (1998), characterized strains of *Salmonella* at an integrated poultry production facility using the RiboPrinter® and found that 97% of samples were given a serotype name with the majority of those isolates coming from samples originating from the processing plant (51%). The RiboPrinter may be capable of identifying samples such as these, which indicates the range of poultry samples collected in the production and processing continuum.

III - RECOVERY AND GENETIC SIMILARITY OF *SALMONELLA* FROM BROILER HOUSE DRAG SWABS VERSUS SURGICAL SHOE COVERS

ABSTRACT

Reducing the contamination of poultry houses requires adequate sampling of the environment. Sampling regimes that assess the *Salmonella* status of broilers prior to processing can be used to identify and process negative flocks first. The current study was designed to evaluate the efficacy of surgical shoe covers versus drag swabs in the recovery of *Salmonella* from poultry house litter. Current methods of determining the colonization status of a flock include drag swabs of the litter surface. This method requires significant time and labor in drag swab assembly preparation. The time and cost involved with drag swab assembly, sterilization, and use decreases the likelihood that the number of samples needed to adequately assess the health of a flock will be taken. A simple solution to the sampling problem is the use of surgical shoe covers. Our results indicate that surgical shoe covers are more effective (12.5%) in recovering *Salmonella* from an infected flock than drag swabs (2.1%). Surgical shoe covers were able to recover three *Salmonella* serotypes while drag swabs recovered only one serotype. The *Salmonella* serotypes from drag swabs that matched the serotypes from surgical shoe covers were genetically identical. Poultry supervisors often travel to farms to assess a flock prior to processing, and to aid in this evaluation, surgical shoe covers may be worn

over disposable plastic boots as supervisors walk the house. Surgical shoe covers cost $0.70 per pair and may be kept in vehicles along with disposable plastic boots.

Keywords: broiler, *Salmonella*, environment, drag swab, surgical shoe covers, litter

INTRODUCTION

In the interest of determining *Salmonella* prevalence in poultry flocks, researchers have sought easy and cost effective methods by which to sample the poultry environment. Environmental sampling has led to control programs in the poultry industry. These programs have reduced the presence of *Salmonella* in breeder flocks and processing plants (Gast and Beard, 1993; Bailey, 1993).

As a means of determining flock colonization status, samples of litter can be taken at random locations in the house and cultured. As an alternative to taking litter samples, a drag swab method was developed to aid in the determination of *Salmonella* prevalence in commercial poultry flocks (Kingston, 1981; Mallinson *et al*., 1989). In an effort to further simplify poultry house litter sampling Caldwell *et al*. (1998) developed a methodology in which protective foot covers were determined to be as effective as drag swabs in isolating *Salmonella* from broiler houses. In a similar approach, researchers in Denmark (Skov *et al*., 1999) demonstrated that, cotton socks worn over boots were found to be as effective in collecting *Salmonella* as compared to fecal samples collected by hand. The objective of the present study was to test for the ability to collect *Salmonella* from the broiler environment under commercial conditions. A secondary objective was to determine if the genetic profile of *Salmonella* collected were similar from both sampling methodologies.

MATERIALS AND METHODS

Experimental design

Two totally cleaned and disinfected commercial broiler farms participated in the study, each with four houses of comparable age, style (dark-out, drop ceiling, and tunnel-ventilated), and size (42 x 500 ft). Total cleanout involved the removal of all old litter, cleaning and disinfection of premises with a non-chlorinated (phosphoric acid and dodecylbenzenesulfonic acid) cleaner, water and feed lines, and application of new litter. Farm A received two flocks of chicks (1 and 2) from broiler breeders that were not vaccinated for *Salmonella*. Farm B (flocks 1 and 2) received chicks from *Salmonella* vaccinated parent stock. Sampling took place from August 2003 through January 2004. Clean peanut hulls were used as the bedding material on both farms. Between each successive flock, caked litter was removed and a top dressing of fresh litter was added in the brooding area. Each farm was sampled three times during two consecutive flocks. The first sample collection was performed on chick delivery day, prior to chick placement. The second collection was midway through growout (~3.5 weeks) and the final collection was on the day of processing.

Sampling procedure

Each drag swab was assembled as described previously (Kingston, 1981; Mallinson *et al*., 1989). Assembled drag swabs were then placed in jars and autoclaved at 121 C for 15 min. Sterile (15 mL) double strength skim milk (Opara *et al*., 1992; Opara *et al*., 1994; Byrd *et al*., 1997) was added aseptically to each jar. New gloves were worn for each house to prevent cross-contamination. Each quadrant of the house was

traversed in a zigzag pattern to ensure proper representation of the area around feed lines, water lines, house mid-section, as well as side areas. Drag swabs were pooled for each house into a sterile Whirl-Pak (product no. B01323; Nasco, Modesto, CA), 50 mL of sterile phosphate buffered saline was added, and the bag was gently massaged prior to placement on ice packs.

Surgical shoe covers (product no. 3688571; KleenGuard Select Protective Apparel, Fisher Scientific, Pittsburgh, PA) were applied aseptically and only worn inside the house. The surgical shoe covers were worn over disposable plastic boot covers (product no. B01323; Nasco, Modesto, CA) and changed out between houses using clean latex gloves. As drag swab sampling was performed, surgical shoe covers were worn so that the sampling pattern and distances matched the pooled drag swabs. Both drag swab and surgical shoe cover sampling was performed in each house during each sample collection. Upon completion of sampling the surgical shoe covers were removed aseptically and placed into a sterile Whirl-Pak; 50 mL of sterile phosphate buffered saline was added, and the bag was gently massaged to ensure even distribution of the media. Samples were then placed in a cooler containing ice packs. All samples were held at 4 C overnight.

Selective enrichment and plating

Selective enrichment was performed according to Caldwell *et al.* (1998) with slight modifications. A volume of 100 mL of tetrathionate-Hajna broth (product no. 0491-17; Difco Laboratories, Detroit, MI) was added to each sample and samples were then incubated for at 37 C for 24 h prior to plating on selective medium. Each sample was streaked onto brilliant green sulfa agar (BGS; Product no. 271710; Difco Laboratories,

Detroit, MI) and double modified lysine iron agar (dmLIA) (MLH, 2003), which were incubated for 24 h at 37 C. Up to four suspect black colonies on dmLIA, or pink colonies on BGS, were subcultured to MacConkey Agar (product no. 212122; Difco Laboratories, Detroit, MI) and incubated at 37 C for 24 h. Characteristic white colonies on MacConkey agar were used to inoculate triple sugar iron (product no. 211749; Becton Dickinson and Company, Sparks, MD) and lysine iron agar slants (product no. 284920; Difco Laboratories, Detroit, MI) which were incubated at 37 C for 24 h.

Automated ribotyping

Cultures producing typical reactions for *Salmonella* were re-streaked onto brain heart infusion (BHI) agar and incubated at 37 C for 24 h in preparation for automated ribotyping. Cultures of *Salmonella* grown on BHI were examined to ensure purity. A small group of cells was selected from the plate and suspended in 200 mL of sample buffer via vortexing. A 30 mL aliquot of this sample was transferred to a single well of an eight well sample carrier. The sample carrier, buffer, and all further reagents were part of a kit designed for the RiboPrinter™ System. The RiboPrinter™ Microbial Characterization System (Qualicon, Wilmington, DE) identified the bacterial genus, species, and subspecies through the analysis of genomic fragments of ribosomal RNA operons (Bruce, 1996; Oscar, 1998). The restriction enzyme *PvuII* (New England Biosystems, Inc., Beverly, MA) was used to differentiate ribotypes and the resulting patterns were analyzed using BioNumerics software (Applied Maths, Austin, TX). This software created a dendrogram of the culture's genetic similarity based on Pearson's coefficient and clustered by an unweighted pair-group arithmetic averaging method.

RESULTS AND DISCUSSION

Each farm was sampled six times. Drag swab and surgical shoe cover samples were obtained from flocks 1 and 2 (n = 96). Over both flocks, more surgical shoe cover samples (12.5%) indicated that a house was positive for *Salmonella* than did drag swab samples (2.1%; P= 0.007; Figure 1). Farm A, during the first flock growout, had no recovery of *Salmonella* for drag swabs or surgical shoe covers. Drag swabs samples of the second flock on Farm A were also *Salmonella* negative but there was a 41.7% rate of recovery for surgical shoe covers (n=5). On Farm A, the average *Salmonella* recovery over two consecutive flocks with surgical shoe covers was 20.8%. Farm B yielded one positive drag swab (flock 2) and one positive surgical shoe cover (flock 1).

On both farms, flock 2 yielded the highest number of *Salmonella* positive houses (n=6). *Salmonella* was recovered from surgical shoe covers when sampling both flocks. Surgical shoe covers yielded *Salmonella* in houses on chick placement day (6.3%), mid-grow out (18.8%), and on processing day (12.5%) (P > 0.05; Figure 2). During flock 2, a drag swab was positive for *Salmonella* at mid-grow out. Drag swabs taken during chick placement day and processing day for both flocks 1 and 2 did not identify any positive houses. House 3 on Farm A was the only house that did not produce positive drag swabs or surgical shoe covers throughout the study (Table 1).

The RiboPrinter™ identified three *Salmonella* serotypes. Surgical shoe covers yielded the greatest number of serotypes and multiple isolates of *Salmonella* including *S.* Kentucky (n=8), *S.* Heidelberg (n=2), and *S.* Meleagridis (n=4). The drag swab sample produced a single isolate of *S.* Kentucky. Both farms carried *S.* Kentucky, but Farm A

also had *S.* Meleagridis, while Farm B had *S.* Heidelberg. The *S.* Kentucky isolates found in the drag swab and surgical shoe covers had the same ribotype.

Cluster analysis indicated three distinct genetic groups corresponding to the three serotypes identified by the RiboPrinter™. All the *Salmonella* isolates combined had a similarity level of 44%. All the *S.* Kentucky isolates were 86% similar, while the *S.* Heidelberg isolates showed greater similarity (91%). The *S.* Meleagridis isolates had the greatest similarity (95%). No patterns based on farm, flock or house could be identified within the *S.* Kentucky isolates. All *S.* Heidelberg and all *S.* Meleagridis isolates came from Farm B and Farm A, respectively (Figure 3).

Given the frequency of *Salmonella* recovery with surgical shoe covers over drag swabs, this method of sampling was more reliable as an indicator of persistent *Salmonella* in the poultry house. Farm A yielded *S.* Kentucky during consecutive visits when surgical shoe covers were employed. Surgical shoe covers were used to identify a higher number of *Salmonella*-positive houses on Farm A than on Farm B. Given the small sample size in this study, caution must be exercised in interpreting the results. Greater repetition in sampling or the addition of more farms to the study would provide more data on which to base stronger statistical support for the use of one method over another.

On chick placement day, the resident strain of bacteria is the most important source of contamination; more so than any other strain that may be introduced later in the growing period (Lahellec *et al*., 1986). Conversely, our samples did not recover resident *S.* Kentucky in the house on chick placement day so it is reasonable to state that the litter was not the source of the organism. Chicks and eggshells from the hatchery (data not shown) were positive on chick placement day and were more likely to have been the

primary source of contamination although breeding adults and vaccine strains can not be ruled out.

Our results concur with other research in that there is very little predictability in *Salmonella* serotype isolation on individual farms over time (Caldwell *et al*., 1995), with the exception of *S.* Kentucky and *S*. Heidelberg. These two strains of *Salmonella* are commonly found in poultry (Hird *et al*., 1993; Byrd *et al*., 1999; Roy *et al*., 2001) and can cause disease in humans (Hird *et al*., 1993). *S*. Kentucky appears to repeatedly have been a contaminant on both farms that persisted over the course of two flocks. Given an expanded sampling period it may have been possible to predict, with the use of surgical shoe covers, the age at which birds begin to shed the organism, providing opportunity for corrective action.

Surgical shoe covers consistently retained a large number of peanut hulls and fecal material during sampling on chick placement day as well as on subsequent visits when birds were older. The effectiveness of recovery may be attributed to the volume of litter and fecal material retained on surgical shoe covers. Multiple drag swabs are more effective than a single drag swab in revealing a higher frequency of *Salmonella* isolation in both vacant and occupied poultry houses (Caldwell *et al*., 1994). Further, it may have been possible to achieve a higher rate of *Salmonella* isolation if surgical shoe covers were changed between quadrants of the house.

The cost of a pair of surgical shoe covers, such as those used in this study, was $0.70 per pair. The cost of sterile, pre-packaged drag swabs can range from $1.56 (product no. TS15-H; Technical Service Consultants, Lancashire, UK) to $2.00 (product no. DS-001; Solar Biologicals, Inc., Ogdensburg, NY). It was also noted that

maneuvering through a house with drag swabs was extremely difficult as birds aged because of their reluctance to move. As a result, it was much easier to obtain a representative sample of the house with the surgical boot covers than with the drag swabs. Industry personnel routinely wear plastic protective foot covers so donning surgical boot covers would not be considered an extraordinary measure. Ease of use and cost both contribute to the feasibility of surgical shoe covers as a sampling method.

Salmonella monitoring of broiler houses using surgical shoe covers results in better recovery than the traditional drag swab method. Surgical shoe covers are less labor intensive to prepare, easy to apply in the field, and less expensive than pre-packaged drag swabs. These findings indicate that surgical shoe covers can yield a far greater number of *Salmonella*-positive samples than drag swabs and is a suitable addition to *Salmonella* monitoring programs.

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Table 1. *Salmonella* serotypes recovered from two farms sampled on the first day through the end of the rearing period during two consecutive flocks.

	Farm A								Farm B							
	Flock 1				Flock 2				Flock 1				Flock 2			
House	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Chick Placement																
Surgical Boots	-	-	-	-	+[C]	-	-	-	-	-	-	-	-	-	-	-
Drag Swabs	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mid-growout																
Surgical Boots	-	-	-	-	+[A]	+[A]	-	+[A]	-	-	-	-	-	-	-	-
Drag Swabs	-	-	-	-	-	-	-	-	-	-	-	-	+[A]	-	-	-
Processing Day																
Surgical Boots	-	-	-	-	-	-	-	+[A]	+[B]	-	-	-	-	-	-	-
Drag Swabs	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[A] *Salmonella* Kentucky, [B] *S.* Heidelberg, [C] *S.* Meleagridis

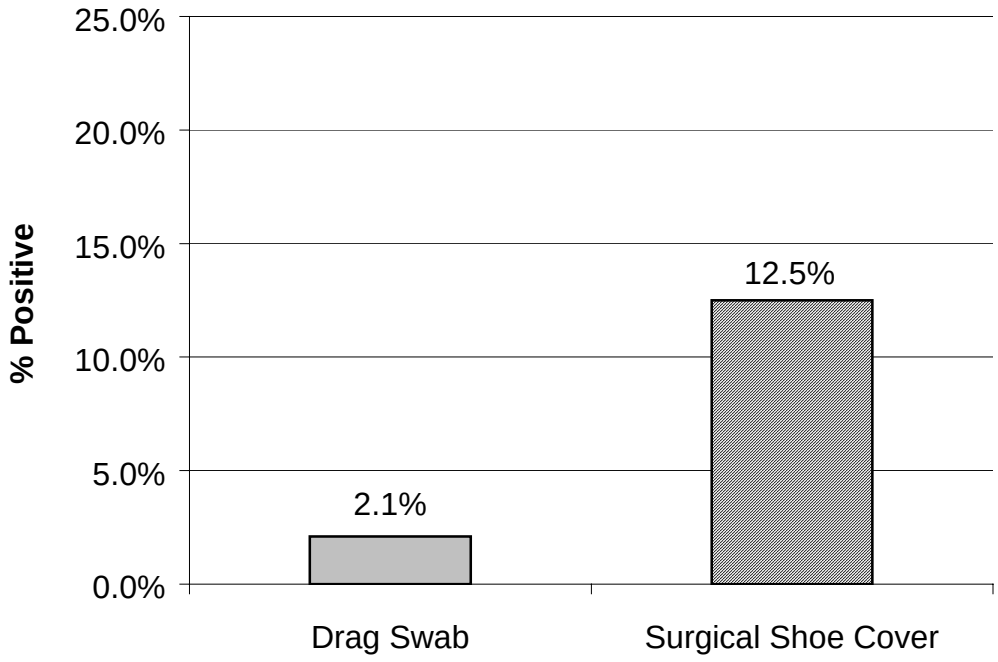

Figure 1. Percentage of *Salmonella* positive drag swab and surgical shoe cover samples from two poultry farms during two consecutive flocks.

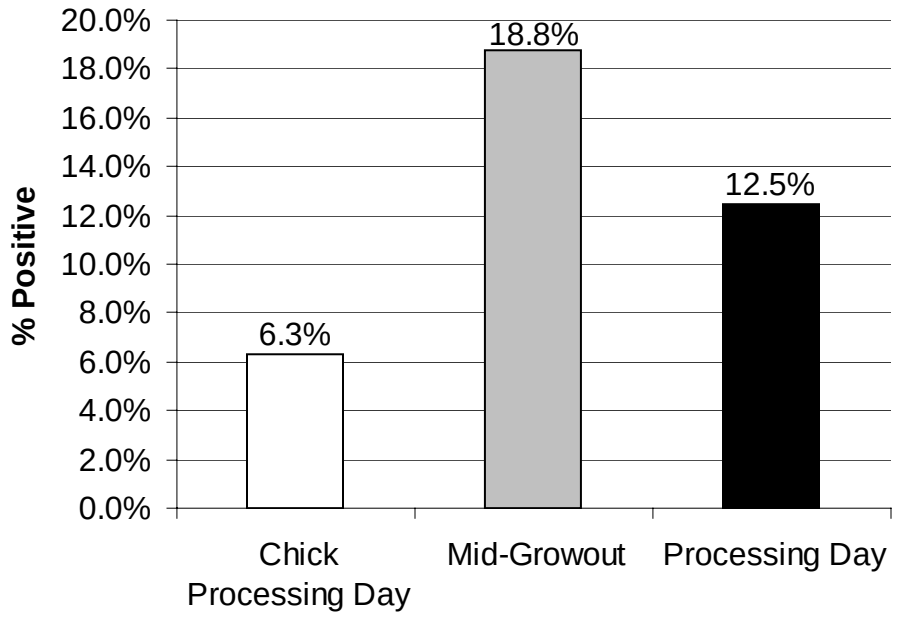

Figure 2. Percent of *Salmonella* positive houses and age of birds at sampling using the surgical shoe cover method during two consecutive flocks.

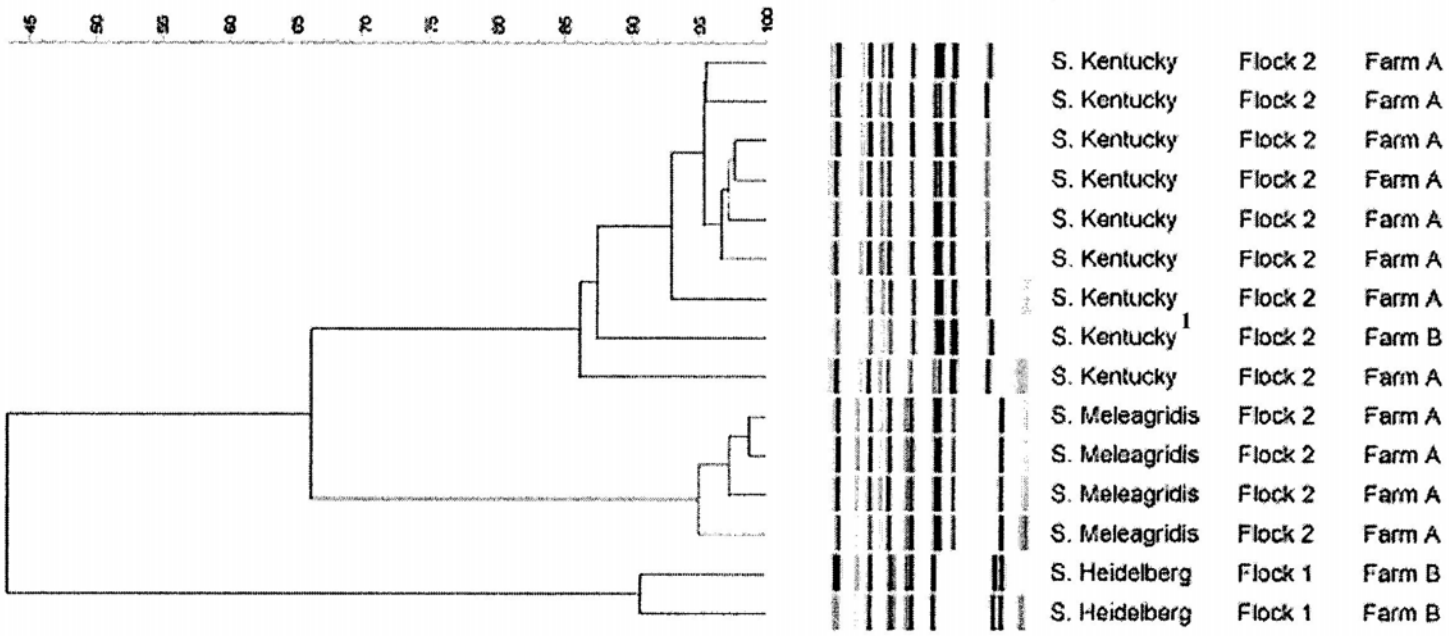

Figure 3. *Salmonella* Dendrogram. Cluster analysis based on Pearson's coefficient indicated three distinct groups with a similarity level of 43%. [1] Drag swab isolate.

IV - RECOVERY AND GENETIC DIVERSITY OF ESCHERICHIA COLI ISOLATES FROM DEEP LITTER, SHALLOW LITTER, AND SURGICAL SHOE COVERS

ABSTRACT

Bedding materials (litter) are a common source of infectious agents in poultry production environments. Because of its heterogeneity, i.e., particle size and composition, it is difficult to obtain representative samples from the litter. Most methods involve surface sampling, i.e., through drag swabs or surgical shoe covers. Surface samples may not represent the true infectious agent content of litter. It is hypothesized that bacteria recovered with surface sampling methods are not representative of the population in the litter. In order to test this hypothesis, both shallow (top 2 in.) and deep (bottom 2 in.) litter samples were taken and compared with a surface sampling method, i.e., surgical shoe covers. *Escherichia coli* (*E. coli*) isolates were recovered from all three sample levels and examined using an automated ribotyping system to determine the genetic diversity of the isolates. Samples were taken of used peanut hull litter over dirt floors during two consecutive flocks on chick placement day, mid-growout, and processing day. Twenty-six unique strains were recovered from the three different sampling methods. Five core isolates (19%) maintained a high frequency of recovery among all three methods. Surgical shoe covers, shallow litter and deep litter samples

recovered 13, 13, and 15 strains, respectively. All strains consisting of two or more isolates, with the exception of one, were found on both farms. There was no correlation among strains between age, house, flock, or farm and ribogroups. One strain, 14176, was found to be clonally related when all isolates were compared, indicating a possible common source such as breeders, hatchery, or feed. Based upon the patterns of *E. coli* recovery among the different sample types, our results indicate that surface sampling methods are equally capable of recovering common isolates from the litter. Surgical shoe covers were easy to use, provided the same core population of isolates, and were comparable to shallow litter in the number of strains recovered.

Key words: *E. coli*, litter, surgical shoe covers, automated ribotyping

INTRODUCTION

The colonization status of a poultry flock can be assessed through samples taken from the environment or from the birds. Food safety begins at the farm with the potential for processing plant scheduling being determined by the pathogen positive or negative status of the flock. As an indicator of fecal contamination, *Escherichia coli* (*E. coli*) is scrutinized in the processing plant and is considered a commensal organism in poultry litter. Poultry litter *E. coli* levels as high as 10^8 cfu/ml occur in the litter (Macklin *et al*., 2005). Litter samples can be obtained by several methods. Grab samples have been replaced by drag swabs as an effective method by which to sample broiler flocks for *Salmonella* (Kingston, 1981; Mallinson *et al*., 1989). Shoe covers are a simpler litter sampling method for broiler houses (Caldwell *et al*., 1998; McCrea *et al*., 2004). Both of these methods allow for greater surface areas to be sampled in comparison to grab samples that are limited by volume and location.

The objective of our study was to test the hypothesis that surface sampling does not allow a true bacterial assessment of the litter composition. We compared litter samples obtained simultaneously from different depths with surgical shoe covers. Bacterial isolates were assessed and compared through the use of automated ribotyping.

MATERIALS AND METHODS

***Experimental Design*.**

Two commercial broiler farms participated in the study, each with four houses of comparable age, style (tunnel-ventilated), and size (42 x 500 ft). The first sample collection was performed on chick delivery day, prior to chick placement. The second

collection was midway through growout (~3.5 weeks) and the final collection was on the day of processing. Houses were top dressed with fresh peanut hulls prior to chick placement in each flock. The litter sampling was conducted on the third and fourth consecutive flocks after the annual total cleanout; taking place between January and May 2004.

Sampling Procedure.

Peanut hulls composed the litter on participating farms, with grab samples being collected from two random locations within each quadrant of the house, then pooled into a sterile plastic bag. Shallow litter samples consisted of grab samples from the top 2 inches of the litter, while deep litter consisted of the bottom 2 inches of the litter. To prevent cross-contamination, latex gloves were changed between deep and shallow litter samples. Samples were placed in an ice chest with ice packs during transport to the laboratory.

Surgical shoe covers[1] were placed aspectically over disposable plastic shoe covers and only worn inside the house. One pair of surgical shoe covers was worn for each house. After sampling, the surgical shoe covers were placed into a sterile whirl-pak containing 250mL of sterile phosphate buffered saline (PBS). Samples were placed in an ice chest during transport to the laboratory.

Laboratory Processing, Culture and Identification

Litter samples were mixed thoroughly and diluted 10:1, based upon a 20g subsample in a sterile stomacher bag, using buffered peptone water (BPW). Samples were then stomached for 1 min. A sterile, cotton-tipped swab was placed in the BPW

[1] no. 3688571; KleenGuard Select Protective Apparel, Fisher Scientific

containing the sample and streaked onto a MacConkey Agar (MA) plate. After streaking for isolation, plates were incubated at 37°C for 18-24 h. Presumptive positive isolates were based on colony color (lactose positive fermentation) and morphology. Up to four colonies from each sample were then subcultured to cryovials containing 0.75mL BPW and incubated overnight at 37°C. A volume of glycerol, 0.75mL, was added to the cryovials, mixed, and then samples were stored at -85°C. Surgical shoe cover samples were mixed in a stomacher for 1 min and all isolation procedures were identical to those of litter samples.

Automated Ribotyping

One isolate from each sample method and house from each sample collection was removed from the freezer and streaked onto Brain Heart Infusion (BHI) agar. Plates were checked for purity prior to automated ribotyping with the RiboPrinter® microbial characterization system[2]. Samples and reagents were prepared according to the manufacturer's instructions. The sample carrier, buffer and all further reagents were members of a kit designed for the RiboPrinter®. Briefly, a small group of cells was selected from the primary quadrant of the plate and transferred to a microcentrifuge tube containing 200µl of buffer. An aliquot of each sample was transferred to a single well of an eight well sample carrier. Samples were heat-treated for 20 min at 80°C, to prevent degradation by endogenous DNA enzymes, prior to the addition of lysis buffer and placement into the RiboPrinter®. Sample DNA was digested with *EcoRI* enzyme followed by agarose gel electrophoresis to separate fragments, then transferred to a nylon membrane. The membrane was hybridized with a chemiluminescent labeled probe

[2] Qualicon, Inc., Wilmington, DE.

containing an rRNA operon (*rrnB*) from *E. coli* (Bruce, 1996). The riboprint pattern of restriction fragments was converted to digital form through the use of a charge-coupled-device camera and stored in the RiboPrinter® database. Analysis of riboprint patterns was performed using BioNumerics[3] software. All riboprint patterns produced by the same restriction enzyme were compared and genetic similarity was calculated based on Pearson's coefficient and clustered by an unweighted pair-group arithmetic averaging method.

RESULTS

The majority, 95.8% (n=46), of surgical shoe cover samples (n=48) yielded *E. coli* suspects. Shallow and deep litter samples (n=48 each) had a slightly lower recovery of *E. coli* suspects, yielding 83.3% (n=40) and 85.4% (n=41), respectively. Each visit to farms yielded *E. coli* for all three sampling methods although not all houses on each visit produced suspect colonies. Of the 127 suspect colonies, 65.4% (n=84) were confirmed as *E. coli* by the RiboPrinter®. Surgical shoe covers, shallow litter and deep litter samples yielded 63% (n=29), 65% (n=26), and 70.7% (n=29) RiboPrinter® confirmed *E. coli* isolates, respectively. Additionally, 1.6% of the *E. coli* suspects were identified as other bacteria (n=2). Unfortunately, 33.0% (n=41) of the *E. coli* suspects were not identified by the RiboPrinter® and were considered unknowns. The status of unknown was conferred upon an isolate if the riboprint pattern was not in the RiboPrinter® database. Further testing of unknown isolates to determine *E. coli* status was not performed.

[3] Applied Maths, Austin, TX.

A total of 26 different strains of *E. coli* were revealed with five strains containing 62% of the confirmed isolates. Deep litter samples yielded the greatest diversity in the number of isolates recovered with 15 strains. Shallow litter and surgical shoe cover samples each recovered 13 strains. The majority of strains, 81% (n=21), yielded less than five isolates. Five strains (19%) yielded more than five isolates and strain number 14147 was found with the greatest frequency (n=14). This same strain was found frequently (93%) in surgical shoe covers and deep litter samples and only one isolate was located in shallow litter. Strain 14176 was fairly evenly distributed among the three sample types. In contrast, shallow litter samples yielded strains 14217 and 14194 more frequently than other samples while deep litter samples yielded 50% of the isolates for strain 18663 (Table 1). Overall, amongst the five most frequently recovered isolates, surgical shoe covers, shallow litter and deep litter samples yielded these isolates in 34.5%, 31%, and 34.5%, respectively, of their total number of isolates. All strains yielding multiple isolates, with the exception of one strain, were found on both farms.

When all isolates were compared, grouping patterns within the dendrogram were randomly distributed between house, farm, age of visit, and flock. Embedded within the dendrogram were two tight clusters of strains. Strain 14176 was 90% homogeneous when compared with isolates from all samples (data not shown). The five most frequently isolated strains were 71% homogeneous. Based upon an 80% similarity level, cluster analysis of the surgical shoe cover, shallow litter, and deep litter samples yielded 7, 6, and 7 groups, respectively. Within the surgical shoe cover samples there was 52.4% homogeneity between all isolates recovered (Figure 1). Within shallow litter isolates,

there was 32.3% homogeneity between isolates (Figure 2) and deep litter isolates had the lowest homogeneity with 11.8% between isolates (Figure 3).

DISCUSSION

Overall, when all three sample types were compared, the tightly clustered strain 14176 fell within its own ribogroup indicating a clonal population. Interestingly, this strain was found in different houses, at different times on both farms, perhaps indicating a common origin beyond the farm such as the breeder population, hatchery, or feed. Common sources of *E. coli* on the farm can include water, dust, vectors, and poorly maintained litter or ventilation systems (Barnes *et al*., 2003). Singer *et al.* (2000) concluded that certain *E. coli* strains can become endemic to the farm environment and infect successive flocks. With the exception of strain 14176, all other strains were randomly distributed indicating individual development rather than clonal spread. These results indicate the capability of automated ribotyping to identify patterns among samples potentially emanating from a common source (Norton *et al*., 2001).

Based upon the patterns of *E. coli* recovery among the different sample types, our results indicate that any of the methods are capable of recovering the most common isolates. Multiple isolates were present for two consecutive flocks and were detectable at multiple sampling ages. Similar results in a study of *Clostridium perfringens* on broiler farms were found regarding multiple isolate ribotypes recovered from several farms and time periods (Craven *et al*., 2000a). Deep litter samples yielded two more strains than surgical shoe covers or shallow litter samples but these strains were not among the most frequently isolated. Surgical shoe covers were easy to use, provided the same core

population of isolates, and were comparable to shallow litter in the number of strains recovered.

Litter sampling remains an excellent method by which to monitor the colonization status of flocks (Mallinson *et al*, 1989), including the anti-microbial resistant *E. coli*. Automated ribotyping is capable of discriminating between fluoroquinolone-resistant *E. coli* (Khan *et al*., 2005). Although automated ribotyping of litter isolates is valuable, there is very little similarity between pathogenic *E. coli* from lesions and *E. coli* from the litter (Jeffrey *et al*., 2004). The lack in variation among our isolates may indicate that broilers are sufficiently mixing the litter to allow for even distribution. This would suggest that surface sampling techniques accurately represent *E. coli* litter flora.

Accurate representations of the litter microflora can be obtained by using surgical shoe covers. The increased genetic diversity of the deep litter isolates could be influenced by the harsher, possibly anaerobic, environment that may or may not contain the same amount of nutrients for the bacteria. The deviation between homogeneities in the surgical shoe cover and shallow litter isolates, given that they each recovered 13 strains, demonstrates that the genetic diversity of *E. coli* recovered from litter remains unpredictable.

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Table 1. Distribution of the five most frequently isolated strains among the three sampling methods.

Strain ID	Surgical Shoe Covers N (%)	Shallow Litter N (%)	Deep Litter N (%)
14147	7 (50.0%)	1 (7.1%)	6 (42.9%)
14176	4 (33.3%)	3 (25.0%)	5 (41.7%)
14217	2 (18.2%)	6 (54.5%)	3 (27.3%)
14194	3 (33.3%)	5 (55.6%)	1 (11.1%)
18663	2 (33.3%)	1 (16.7%)	3 (50.0%)

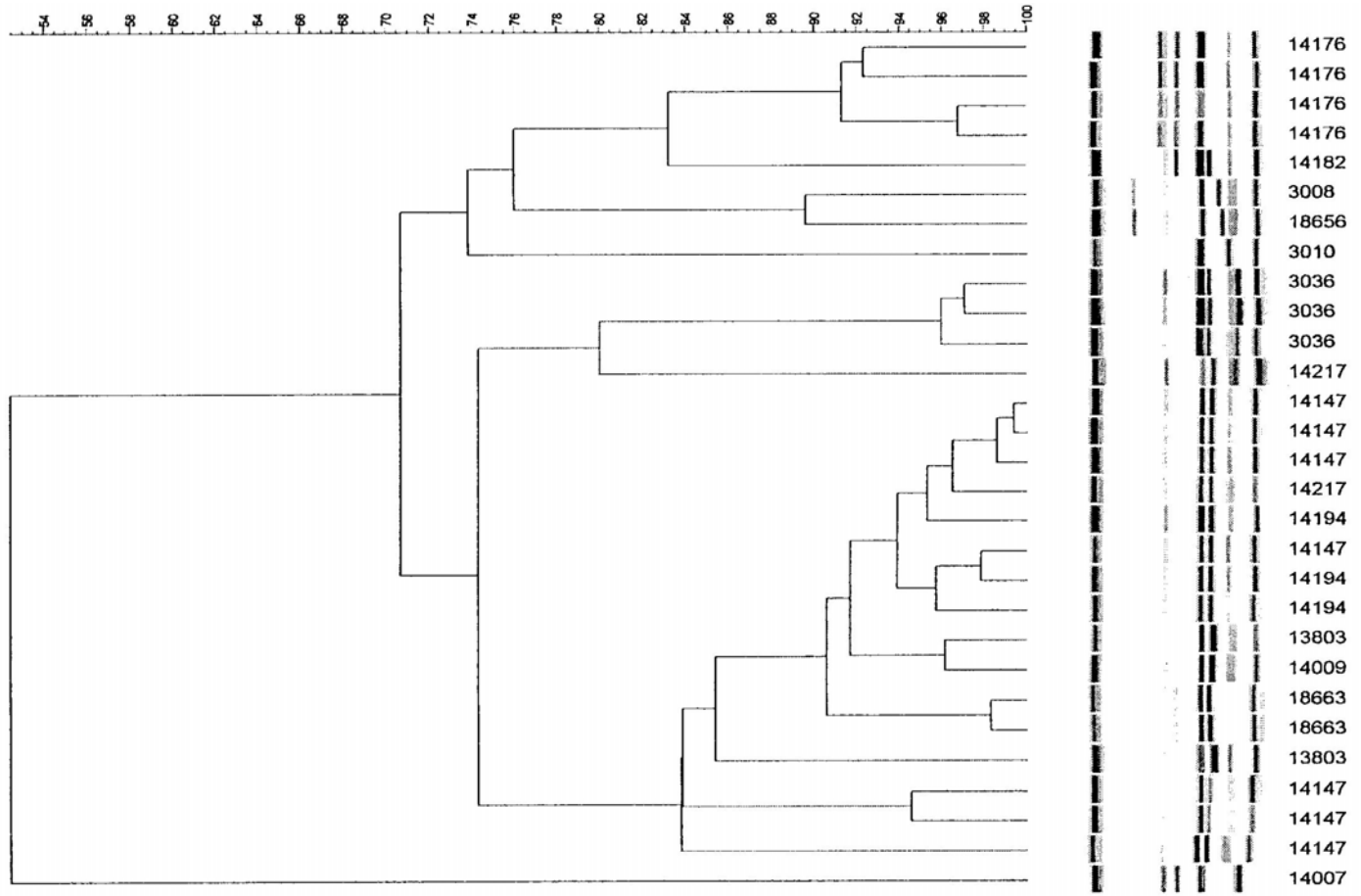

Figure 1. Ribotypes of surgical shoe cover *E. coli* strains. Numerical analysis of 16S rRNA by *EcoRI*. Clusters show the patterns of all types obtained. Numerical analysis of the 29 patterns are presented as dendrograms.

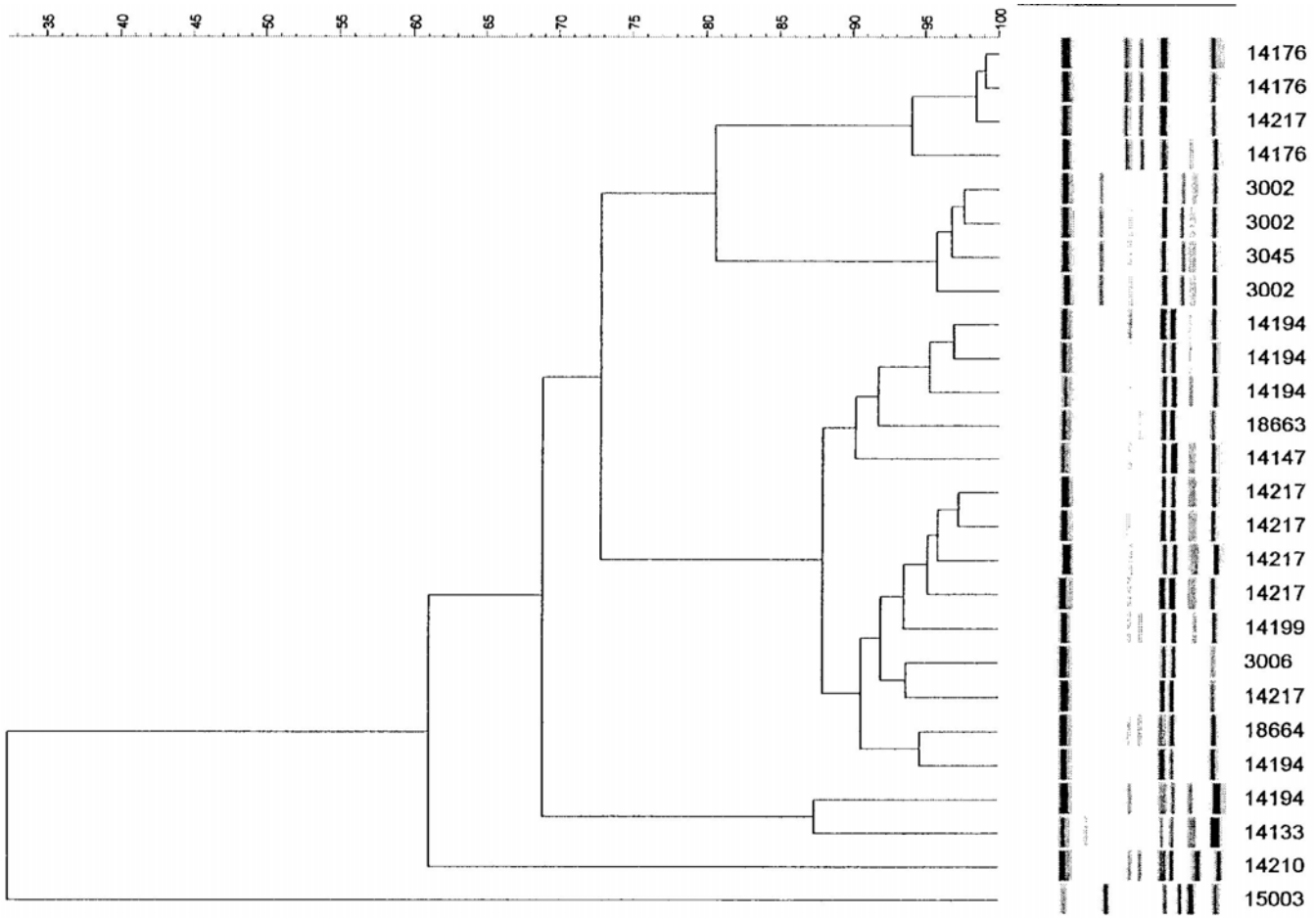

Figure 2. Ribotypes of shallow litter *E. coli* strains. Numerical analysis of 16S rRNA by *EcoRI*. Clusters show the patterns of all types obtained. Numerical analysis of the 26 patterns are presented as dendrograms.

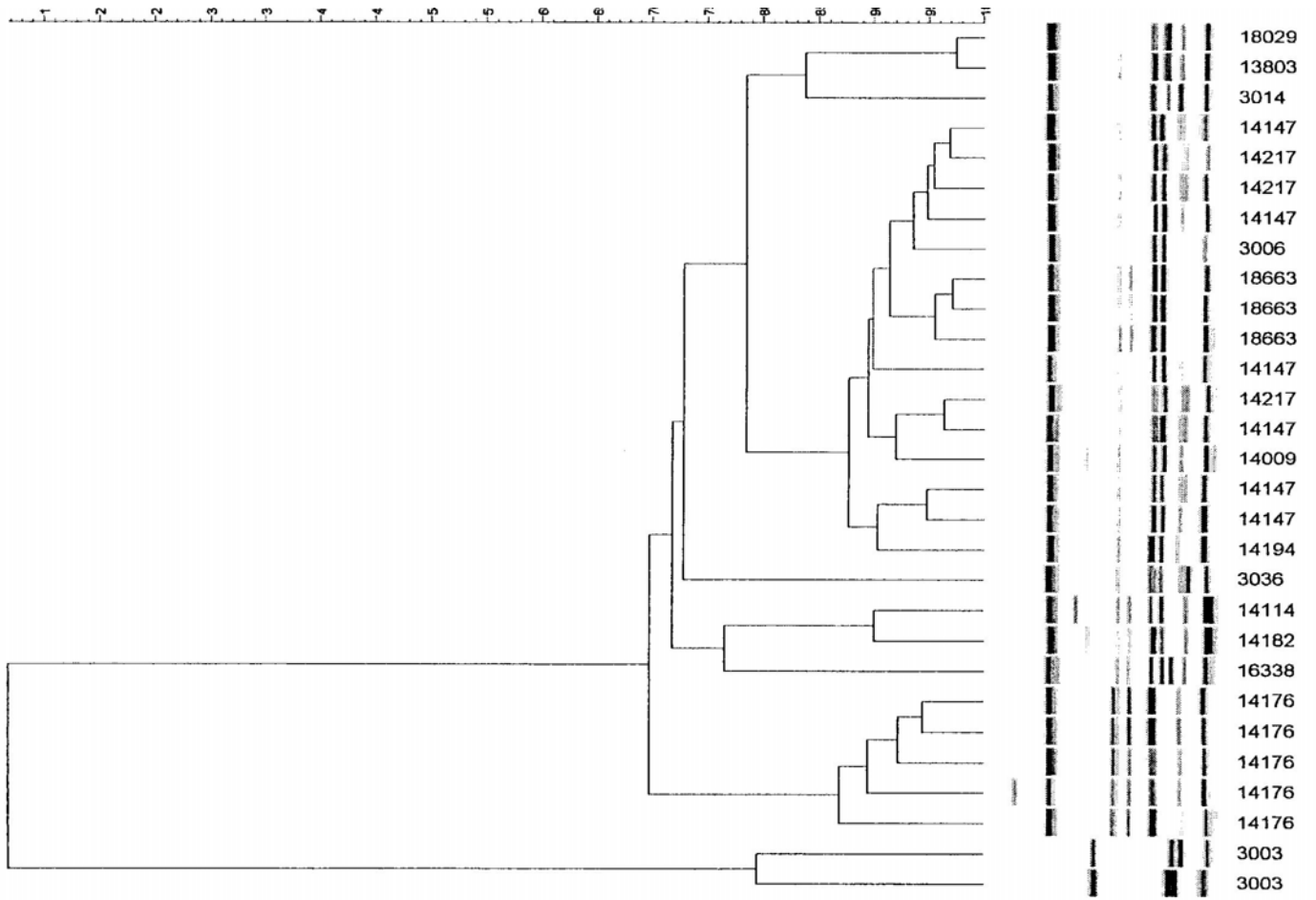

Figure 3. Ribotypes of deep litter *E. coli* strains. Numerical analysis of 16S rRNA by *EcoRI*. Clusters show the patterns of all types obtained. Numerical analysis of the 29 patterns are presented as dendrograms.

V - A LONGITUDINAL STUDY OF *SALMONELLA* AND *CAMPYLOBACTER JEJUNI* ISOLATES FROM DAY OF HATCH THROUGH PROCESSING USING AUTOMATED RIBOTYPING

ABSTRACT

Comparisons of bacterial populations over long periods of time allow researchers to identify clonal populations, perhaps those responsible for infection of multiple farms or humans. *Salmonella* and *Campylobacter* can cause human illness and our objective was to use a library typing system to track strains that persist in the poultry house and through the processing plant. Two farms, over four consecutive flocks, were studied. Multiple samples were taken of the poultry house environment, feed mill, transport crates, and carcasses in the processing plant. Sample collection on the farm took place on chick placement day, mid-growout, and on processing day.

This study found 80.3% of isolates belonged to a single strain of *Salmonella* Kentucky that persisted in several environmental samples for all flocks, at both farms, from chick placement day to the final product at the plant. Surgical shoe covers produced the majority (n=26) of isolates and processing day yielded the highest recovery (n=68). Additional serotypes were recovered, but the *S.* Kentucky-positive eggshells and chick mortality appeared to be the source of the organism for both farms given the clonal population.

All *Campylobacter* isolates recovered were identified as *C. jejuni*. The majority of *Campylobacter* isolates (90.1%) belonged to one of three core strains. *C. jejuni* was not recovered on chick placement day and cecal droppings yielded all nine strains. The majority (98.2%) of isolates were from Farm A. Cluster analysis grouped all *C. jejuni* and *Salmonella* based upon a similarity level of 55.9% and 43.9%, respectively.

Key words: Salmonella, Campylobacter, automated ribotyping, environment, processing.

INTRODUCTION

Salmonella and *Campylobacter* are the pathogens most responsible for human foodborne gastroenteritis in the United States. In 2004, nearly 6,500 cases of salmonellosis and over 5,600 cases of campylobacterosis were diagnosed. *Salmonella* and *Campylobacter* may be responsible for illness in as many as 14.7 and 12.9 cases, respectively, out of every 100,000 persons (CDC, 2005). Both organisms are strongly associated with poultry products and these products have been implicated in outbreaks of foodborne disease (Gast, 2003; Shane and Stern, 2003). Pre-harvest control of *Campylobacter* is now the focus of pre-harvest pathogen reduction programs in hopes of decreasing the bacterial load that enters the plant (Shane and Stern, 2003).

Biosecurity is a mindset, as well as a set of good manufacturing practices for the farm. Effective cleaning and disinfection programs, in conjunction with a biosecurity plan, is capable of reducing or eliminating problematic pathogens. Longitudinal sampling of multiple flocks can elucidate the seasonality of bacterial prevalence patterns as well as cleaning and disinfection efficiency (Berndtson *et al.*, 1996b). Seasonal preferences have been identified for *Campylobacter* with prevalence rates increasing during the warm summer months (Jacobs-Reitsma *et al.*, 1994; Hudson *et al*., 1999; Smitherman *et al.*, 1984; Pearson *et al.*, 1996; Annan-Prah and Janc, 1988).

When examining poultry production on a farm to fork basis there are multiple variables to consider. Pathogen reservoirs within the poultry house environment include litter, water, feed, vectors, dust, and poorly cleaned equipment (Dawe, 2005; Shane and Stern, 2004; Gast, 2003). Broiler chickens may arrive at the house infected with *Salmonella* acquired at the hatchery (Bailey *et al.*, 1994; Cox *et al.*, 1990) or through

infected breeding stock (Gast, 2003). Paratyphoid *Salmonella* infections of broilers affect chickens during the first two weeks of age but resistance in chickens increases with age (Gast, 2003) while *Campylobacter* colonizes chickens older than 3 weeks (Shane and Stern, 2003). Once introduced to a flock, *Campylobacter* can move rapidly to infect the flock as a whole utilizing the copraphagic nature of chickens and generalized horizontal transmission. Shortly after the identification of *Campylobacter* in flocks, the prevalence may reach 90-100% (Shane, 2000).

Feed does not tend to harbor *Campylobacter* because of the low moisture content of feed along with the organism's intolerance to desiccation (Doyle and Roman, 1982). Conversely, feed can be a route of infection for *Salmonella*, especially feeds that contain animal proteins and are not heat treated (Gast, 2003). Non-pelleted feeds may be contaminated more often than pelleted feeds (Morris *et al.*, 1969). *Salmonella* numbers are reduced by the high temperatures used in the pelleting process (Rinehart, 1994; Jones *et al.*, 1991).

Water is an excellent conveyor of the *Campylobacter* organism on broiler farms (Lindblom *et al.*, 1986; Gregory *et al.*, 1997; Pearson *et al.*, 1993; Smitherman *et al.*, 1984). *Campylobacter* can survive in water but may not necessarily be the site of amplification. Infected chicks placed in isolator units were able to contaminate waterers but once the chicks were removed, the *C. jejuni* levels in the waters decreased significantly within a 48 hour period (Shanker *et al.*, 1990). Water is also an excellent source of horizontal transmission of the *Salmonella* organism. In a study by Nakamura *et al.* (1994) *Salmonella* inoculated hens were able to infect uninoculated hens sharing the same water source within one day.

The stress of transportation increases the prevalence of *Salmonella*- and *Campylobacter*-positive broilers entering the processing plant due to the fecal contamination of skin and feathers by adjacent broilers (Stern *et al.*, 1995). The potential exists for bacteria from the farm to remain on a carcass despite control measures in the processing plant (Berndtson *et al.*, 1996a). *Salmonella* was detected on 43% of ready-to-cook broiler carcasses (Bokanyi *et al.*, 1990) while *Campylobacter* was detected by culture on up to 100% of processed poultry (Stern, 1995). Current methods of processing broilers can increase the contamination of carcasses with *Salmonella* and *Campylobacter* as they are placed on the shackles to the final product (Mead *et al.*, 1994; Oosterom *et al.*, 1983; Wempe *et al.*, 1983). Within the processing plant points of cross-contamination include the scald tank, feather picking machine, and chill tank (Wempe *et al.*, 1983). Izat *et al.* (1988) noted that the isolation rate of *Campylobacter* on carcasses or equipment can be dependent upon the processing plant sampled, thus plants within a state or country may yield differing results.

Those bacterial isolates that remain over multiple flocks on both farms may indicate clonal spread due to a common origin (Norton *et al.*, 2001). Using molecular characterization techniques, such as automated ribotyping, these isolates may be identified and stored in a database for use in longitudinal studies aimed at identifying problematic strains of bacteria. This objective of this study was to examine *Campylobacter* and *Salmonella* isolates gathered from two poultry farms over one year and compare them with isolates gathered from the same flocks as they were processed using an automated ribotyping system. Another objective of this study was to use an automated ribotyping system to elucidate the movement of *Salmonella* and

Campylobacter through all phases of growout and processing in an integrated broiler operation during consecutive flocks. We hoped to identify potentially fastidious organisms that could remain in the poultry house environment from flock placement through to the final product in the processing plant. Correlation of isolates obtained from the processed product to sources in the poultry house would aid in implementing a pre-harvest food safety program. Additionally, an examination of flocks over several seasons could identify potential seasonal variations exhibited by some strains.

MATERIALS AND METHODS

Experimental Design

Two commercial broiler farms participated in the study, each with four totally cleaned and disinfected houses of comparable age, style (dark-out, drop ceiling, and tunnel-ventilated), and size (42 x 500 ft). Total cleanouts involved the removal of all old litter (bedding, feces, dander, and insects), cleaning and disinfection of premises, water and feed lines, and application of new litter. Four consecutive flocks were sampled from August 2003 through May 2004. The first sample collection was performed on chick delivery day, prior to chick placement. The second collection was midway through growout (~3.5 weeks) and the final collection was on the day of processing. On the day of processing, four transport containers were sampled by drag swabs after the broilers were delivered to the processing plant. Five carcass rinses were obtained at each of three stations in the plant as the specified flocks were processed. The first station was located prior to carcasses entering the chill tank (pre-chill tank), the second station was just after the carcasses exited the chill tank (post-chill tank), and the third station was immediately

after carcasses exited the anti-microbial dip (post-disinfectant). During the last flock, transport crate swabs and carcass rinses were not obtained from participating farms due to a change in company ownership. Samples of feed and ingredients delivered to each farm were obtained from the feed mill once during each growout.

Sample collection: litter, drag swabs, and surgical shoe covers.

The methods used to collect these samples are described in Section III but the intention in this study was the inclusion of *Campylobacter*. Peanut hulls composed the bedding on participating farms, with grab samples being collected from two random locations within each quadrant of the house, then pooled into a sterile plastic bag. Samples were placed in an ice chest with ice packs during transport to the laboratory. Each drag swab was assembled according to the designs set forth in prior publications (Kingston, 1981; Mallinson *et al.*, 1989; Caldwell *et al.*, 1998). Assembled drag swabs were placed in jars and autoclaved at 121^{o}C for 15 min followed by the aseptic application of 15 ml sterile double strength skim milk (Opara *et al.*, 1992; Opara *et al.*, 1994) before being used in the field. Drag swabs were pooled for each house into a sterile plastic sample bag containing 50 ml of sterile phosphate buffered saline (PBS). Surgical shoe covers[1] were placed aspectically over disposable plastic shoe covers and only worn inside the house. One pair of surgical shoe covers was worn for each house. After sampling, the surgical shoe covers were placed into a sterile whirl-pak containing 250mL of sterile phosphate buffered saline (PBS). Surgical shoe covers were worn as drag swabs were performed in the house to ensure that the same distances were traveled.

[1] no. 3688571; KleenGuard Select Protective Apparel, Fisher Scientific

Sample collection: feed.

Feed grab samples were taken from each of the two feed hoppers in a house and pooled. Both the ingredient and feed samples from the feed mill were placed in sterile plastic bags and sealed for transport to the laboratory. Feed samples included starter, grower, and finisher feeds. Ingredients included soy meal, corn (whole and ground), rice meal, phosphorous, limestone, and poultry meal.

Sample collection: water.

A total of one liter of water was pooled into a sterile glass bottle from four water lines within each house. In addition to water from within the poultry house, standing water was collected from areas around the houses and one liter of water was collected from the wellhead.

Sample collection: insects.

Adult litter beetles (*Alphitobius diaperinus*) and larvae were collected from plastic tubes containing rolled-up paper. The paper and the associated insects were placed into a sterile plastic sample bag and sealed. Insects were held at –80^{o}C overnight before sifting out debris.

Sample collection: miscellaneous.

On chick delivery day, chicks that died in transport (chick mortality) were collected as chicks were placed. Up to 20 chicks were collected per house. Chick mortality for each house was pooled into a sterile plastic bag and sealed. Eggshell fragments were also pooled into a sterile sample bag for each house. Sterile cotton swabs were also used to collect freshly voided cecal droppings from the floor throughout the poultry house. A total of 10 cecal droppings were sampled and pooled in 50 ml of PBS.

Sterile cotton swabs were also used to sample feed equipment, walls, and fans. One tube containing 5 ml of sterile double strength skim milk was used as *Salmonella* sample transport and one tube containing 5 ml of PBS was used for *Campylobacter* sample transport. All samples were placed in an ice chest during transport to the laboratory.

Sample collection: feed equipment, fans, and walls.

Sterile cotton swabs were used to sample the surface of feed equipment, fan shutters, and walls. Each house contained two feed lines. A single feed pan on each line was randomly selected for sampling. The outer surface and dividers, taking care to avoid the feed, on each pan was swabbed. Dust was collected from up to two fan shutters. A randomly selected section of wall was swabbed at broiler height. Each swab was immediately placed into a tube containing transport media.

Sample collection: transport crates and carcass rinses.

At the processing plant, transport crates were swabbed, using a 4 x 4 inch piece of sterile gauze. The gauze was then placed into a sterile, plastic sample bag and wetted with 10 ml of sterile PBS. Within the plant, five carcass rinses with 400 ml of buffered peptone water were performed at each of three stations within the processing plant according to the procedure described in the Microbiological Laboratory Handbook (2003b). All samples were placed in an ice chest during transport to the laboratory.

Bacterial Isolation.

Litter, insect, feed, and feed ingredient samples were mixed thoroughly and a 20 g subsample was placed in a sterile stomacher bag. A 10:1 dilution of Tetrathionate-Hajna broth (TTB) and blood-free Bolton's broth (BB) was used for *Salmonella* and *Campylobacter* enrichment, respectively. Samples were then processed in a stomacher

for 1 min. TTB enriched samples were incubated at 37°C for 18-24 h and BB enriched samples were incubated microaerophilically for 48 h at 42°C. After incubation, samples were streaked for isolation on their respective selective agar medias using a sterile, cotton-tipped swab.

Transport crate swabs, drag swabs and surgical shoe cover samples were cultutred by direct plating onto selective media using a sterile cotton swab. Samples were then weighed and a 1:10 dilution of TTB was added prior to aerobic incubation at 37°C for 18-24 h. After incubation, samples were streaked for bacterial isolation on their respective selective agar medias using a sterile, cotton-tipped swab.

Chick mortality from each house were examined at necropsy and ceca were aseptically collected. The ceca were pooled into a sterile plastic bag for each house and 50 ml of sterile BPW was added. Additionally, eggshells from each house were collected and 50 ml of sterile BPW was added. Samples were processed in a stomacher for 1 min. Samples were then cultured by direct plating on selective media using a sterile cotton swab. A 10:1 dilution of TTB was added prior to aerobic incubation at 37°C for 18-24 h. After incubation, samples were streaked for bacterial isolation on their respective selective agar medias using a sterile cotton swab.

All water samples were passed through a sterile 0.22µm filtration unit. The filters were then aseptically removed, sectioned, and placed into 10ml of TTB and BB for the selective enrichment of *Salmonella* and *Campylobacter*, respectively. *Campylobacter* enriched samples were incubated for 48 h at 42°C under microaerophilic conditions and *Salmonella* enriched samples were incubated aerobically for 18-24 h at 37°C. Samples were then streaked for bacterial isolation on their respective selective agar medias.

Campylobacter swabs for fan, wall, and feed equipment swabs were placed into a 1:10 dilution (v/v) of BB and incubated microaerophilically for 48h at 42^{o}C. Samples were cultured by direct plating onto selective media. For the *Salmonella* tubes, a 1:10 dilution (v/v) of TTB was added to the tubes and samples were incubated 18-24 h at 37^{o}C. Samples were then streaked for bacterial isolation on selective media.

Selective Media.

The selective media for *Campylobacter* isolation was CampyFDA agar plates. CampyFDA plates were incubated for 48 h at 42^{o}C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2). The selective medias for *Salmonella* isolation were modified Lysine Iron agar (mLIA) and Brilliant Green Sulfa (BGS) agar plates used for *Salmonella* isolation. BGS and mLIA plates were incubated aerobically for 18-24 h at 37^{o}C.

Bacterial Identification.

Presumptive positive *Salmonella* isolates were based on colony color and morphology. Up to four colonies from each sample were then subcultured on MacConkey agar and incubated for 18-24h at 37^{o}C. Cultures were observed to verify lack of lactose fermentation. Those samples were then streaked onto Triple Sugar Iron (TSI) and Lysine Iron agar (LIA) slants and incubated 18-24h at 37^{o}C. Samples with characteristic reactions on these biochemical tests were then subcultured to cryovials containing 0.75mL BPW. The cryovials were incubated overnight at 37^{o}C. A volume of glycerol, 0.75mL, was added to the cryovials, mixed, and samples then were stored at -85^{o}C.

Presumptive positive *Campylobacter* isolates were based on colony color and morphology. Up to four colonies from each sample were subcultured on Blood Agar

(BA) and incubated microaerophilically for 48 h at 42°C. Colonies exhibiting characteristic growth were subjected to Gram stain, catalase and oxidase testing. Colonies were also examined for characteristic motility via phase-contrast microscopy. These isolates were then harvested from BA plates using a sterile plastic loop. Isolates were immediately placed into cryovials containing 1.5 ml of a 75% PBS: 25% glycerol solution. Samples were then mixed and stored at -85°C.

Automated Ribotyping

One isolate from each sample method and house from each sample collection was removed from the freezer and streaked onto Brain Heart Infusion (BHI) agar (*Salmonella* isolates) or BA plates (*Campylobacter* isolates). Plates were checked for purity prior to automated ribotyping with the RiboPrinter® microbial characterization system[2]. Samples and reagents were prepared according to the manufacturer's instructions. The sample carrier, buffer and all further reagents were members of a kit designed for the RiboPrinter®. Briefly, a small group of cells was selected from the primary quadrant of the plate and transferred to a microcentrifuge tube containing 200µl of buffer. An aliquot of each sample was transferred to a single well of an eight well sample carrier. Samples were heat-treated for 20 min at 80°C, to prevent degradation by endogenous DNA enzymes, prior to the addition of lysis buffer and placement into the RiboPrinter®. Sample DNA were digested with *EcoRI* enzyme followed by agarose gel electrophoresis to separate fragments, then transferred to a nylon membrane. The membrane was hybridized with a chemiluminescent labeled probe containing an rRNA operon (*rrnB*) from *E. coli* (Bruce, 1996). The riboprint pattern of restriction fragments was converted

[2] Qualicon, Inc., Wilmington, DE.

to digital form through the use of a charge-coupled-device camera and stored in the RiboPrinter® database. Analysis of riboprint patterns was performed using BioNumerics[3] software. All riboprint patterns produced by the same restriction enzyme were compared and genetic similarity was calculated based on Pearson's coefficient and clustered by an unweighted pair-group arithmetic averaging method.

RESULTS

Campylobacter

A total of 111 isolates of *Campylobacter jejuni* were recovered from the four flocks. Nine strains of *C. jejuni* were identified. The majority (98.2%) of isolates were from Farm A while Farm B produced only two isolates. *C. jejuni* was never detected on chick placement day during any flock. Three core strains made up 90.1% (n=100) of all isolates obtained and were only found on Farm A. The remainder of isolates (9.9%) clustered into a ribogroup containing six strains. The six strains were identified on processing day, mainly in cecal droppings (n=10), during flocks three and four. The exceptions were the two isolates, each a different strain, recovered from Farm B during mid-growout. The majority of all isolates (70.3%) were recovered on processing day while the remainder (29.7%) were recovered from the mid-growout sample collection of flock two. All feed and ingredient samples obtained from the mill were free of *C. jejuni*. Three strains, composed of 20 isolates, were collected from transport crates while a total of 12 isolates were collected from inside the processing plant. Three strains were

[3] Applied Maths, Austin, TX.

identified inside the processing plant with two strains (n=11) from pre-chill tank samples and one strain (n=1) isolated from a post-chill tank sample.

Samples that yielded isolates included drag swabs, surgical shoe covers, cecal droppings, feed equipment, transport crates, pre-chill tank carcass rinsate, and post-chill tank carcass rinsate (Table 1). Cecal droppings identified all the strains (n=9) while drag swabs, surgical shoe covers, feed equipment, and transport creates each identified the three core strains. Two strains, 2050 and 1141, were found at the pre-chill tank station in the processing plant and strain 2062 was cultured from post-chill tank carcass rinsate. Of the core isolates, the distribution was as follows: 61% strain 2050, 29% strain 1141, and 10% strain 1146. The distribution of the core isolates among the different sample types is demonstrated in Table 2. No core isolates were recovered during mid-growout of flocks one, three, or four. The majority of core isolates (n=31) were found during the mid-growout sample collection of flock 2.

Analysis of the Riboprinter results indicated that isolates, particularly the core isolates, were randomly distributed among houses, time of sample collection, flocks, and source (Figure 1). Cluster analysis grouped all *C. jejuni* based upon a similarity level of 55.9%. Four distinct clusters were obtained when analysis was based upon an 85% similarity level. The core isolates formed the largest cluster based upon a similarity level of 79.6%. A single core isolate formed a separate cluster as did the isolates recovered from Farm B. The last cluster consisted of a variety of intermittent strains obtained on processing day, mainly from cecal droppings, during flock four.

Salmonella

A total of 148 isolates within seven different serotypes of *Salmonella* were recovered from four flocks. The serotypes recovered included *S.* Kentucky (n=119), *S.* Heidelberg (n=21), *S.* Meleagridis (n=4), *S.* Schwarzengrund (n=1), *S.* Schwarzengrund / Bredeney (n=1), *S.* Tennessee (n=1), and *S.* Hadar (n=1). *S.* Kentucky composed the majority (80.3%) of the isolates, all of which were the same strain as identified by the RiboPrinter. Four serotypes, *S.* Kentucky (1 strain), *S.* Heidelberg (2 strains), *S.* Meleagridis (1 strain), and *S.* Hadar (1 strain), were from Farm A. Three serotypes, *S.* Kentucky (1 strain), *S.* Heidelberg (3 strains), and *S.* Tennessee (1 strain), were from Farm B. Only the *S.* Heidelberg sample carried more than one strain during the four flocks. The feed mill poultry meal ingredient sample, obtained during flock four due to a change in feed formulation, yielded *S.* Schwarzengrund and *S.* Schwarzengrund / Bredeney. All other feed mill ingredients were free of *Salmonella*. Chick processing day, mid-growout, and processing day produced 26, 37, and 68 isolates, respectively. Flocks one and two on both farms identified 50 and 63 isolates while flocks three and four produced 26 isolates each. The majority (n=67) of *Salmonella* isolates were obtained during flocks one and two on Farm A, which received chicks from unvaccinated breeders. Flocks three and four on Farm A yielded 33 isolates while a total of 48 isolates were obtained from Farm B.

All samples from the feed mill, farm environment, transport crates, and processing plant, with the exception of post-chill tank carcass rinsate, produced *Salmonella* isolates. Among samples taken on the farm, surgical shoe covers were able to recover all five serotypes and had the highest recovery of isolates (n=26). Cecal droppings and insects

yielded 21 isolates apiece while transport crates and pre-chill tank carcass rinsate each produced 16 isolates. The distribution of the isolates among the different serotypes and samples is demonstrated in Table 3.

On chick placement day, during the first two flocks, the environment of all poultry houses remained free of *S.* Kentucky and *S.* Heidelberg. An exception to this pattern was the recovery of *S.* Meleagridis from one surgical shoe cover sample from flock two on Farm A. Conversely, chick mortality and eggshells in the first two flocks carried *S.* Kentucky into the house. Either the eggshells, chick mortality, or both in all four flocks were positive for *Salmonella*. During flock three, insects were positive for *S.* Heidelberg on chick placement day, possibly making them the source of the identical serotype and strain recovered from surgical shoe covers on the same farm and house during mid-growout and processing day sample collections. Surgical shoe covers produced a similar number of isolates during each flock (6, 8, 6, and 7, respectively) but recovery was greatest during mid-growout sample collections (n=14). Additionally, during mid-growout, litter samples recovered 11 *S.* Kentucky isolates. On processing day, samples with the highest recovery of *Salmonella* isolates were transport crates (n=17), pre-chill tank carcass rinsate (n=16) and cecal droppings (n=15).

Standing water did produce two *S.* Kentucky isolates during chick placement day during flock three on Farm A. This sample was collected from pooled water in front of culverts between poultry houses. One drag swab, water, and feed equipment swab each provided an *S.* Kentucky isolate. Feed samples did provide *S.* Kentucky, but it was noted by researchers that rodents were seen in feed hoppers and could have been the source of infection. Transport crates consistently provided *Salmonella*, as did pre-chill tank carcass

rinsate samples. Conversely, one of five carcasses was positive after the antimicrobial dip in the processing plant during flock one.

Analysis of the RiboPrinter® results indicated that isolates within serotypes were randomly distributed among houses, time of sample collection, flocks, and source (Figure 2). An exception was *S.* Heidelberg which was often recovered in house four of Farm B. Cluster analysis grouped all *Salmonella* isolates based upon a similarity level of 43.9%. Six distinct clusters, founded on serotype, were obtained when analysis was based upon an 80% similarity level. Among those clusters, *S.* Kentucky formed the largest cluster based upon a similarity level of 82.4%. *S.* Heidelberg, *S.* Meleagridis, and both feed mill isolates formed closely related clusters with 88.9%, 94.9%, and 98.2% similarity within their serotypes, respectively. *S.* Tennessee and *S.* Hadar, although single isolates, were intermediaries in their genetic relationship to the large *S.* Kentucky cluster and the smaller clusters of feed mill, *S.* Meleagridis, and *S.* Heidelberg isolates.

DISCUSSION

Campylobacter

It is well-established that natural *Campylobacter* colonization in chickens does not occur before two to three weeks of age. Berndtson *et al.* (1996a) tested 24 flocks of broilers and found no chickens to be colonized at one week old. Engvall *et al.* (1986) found that the house environment, as well as chickens one to two weeks of age, were negative but colonization did occur by three to five weeks of age. Neill *et al.* (1984) found chickens of an older age to be colonized in one study but the same chickens had not been colonized at the age of one week. Similarly, in a study of turkey poults and

eggs, negative results indicated that breeders were not the source of colonization (Acuff *et al.*, 1982). Our study did not yield any *Campylobacter* from environmental samples, eggshells, or chick mortality during chick placement day during any flock. The likelihood of detecting *Campylobacter* in flock or environmental samples during chick placement day or the first two weeks is very small.

As flock age increases, there is a positive correlation with *Campylobacter* recovery (Berndtson *et al.*, 1996a; Jacobs-Reitsma *et al.*, 1995; Kaino *et al.*, 1988; Smitherman *et al.*, 1984). In one study, almost 100% of participating flocks were positive at market age of 49 days (Gregory *et al.*, 1997). Our results concur with previous research that detection in cecal droppings and swabs of the environment increased as the flock matured. Once detected, colonization of the whole flocks is rapid, often occurring within a week (Lindblom *et al.*, 1986; Neill *et al.*, 1984; Smitherman *et al.*, 1984).

Campylobacter prefers to colonize the mucus-filled crypts of the ceca and large intestine of poultry (Beery *et al.*, 1988). A dose of 3500 cfu is sufficient to colonize the ceca of chicks (Stern *et al.*, 1988). Gregory *et al.* (1997) also found that cecal droppings yielded 100% recovery by 49 days in three of four houses. We were able to recover the highest number of *Campylobacter* strains using cecal droppings. This points to the effectiveness of the sampling method when testing for *Campylobacter* colonization in older flocks. Perhaps the addition of surgical shoe covers, which revealed the core *Campylobacter* strains, to cecal dropping samples would be a good combination for testing both flock colonization and environmental status.

The core isolates were not evenly distributed among farms. The fact that Farm B did not yield many isolates indicates that the management of this farm may have been different. The use of enrichment may also have preferentially selected for certain subtypes.

The molecular characterization of the core strains identified a closely related, and perhaps clonal population, of isolates (Norton *et al.*, 2001). It is not uncommon to find more than one strain of *Campylobacter* on the farm (van de Giessen *et al.*, 1992; Pokamunski *et al.*, 1986). Longitudinal studies such as this are capable of elucidating trends among poultry pathogens that would otherwise remain undetected. Library typing methods, such as automated ribotyping, are an excellent way to typify and catalog endemic and epidemic strains of *Campylobacter* and *Salmonella* in poultry and poultry products (Manfreda and De Cesare, 2005; Oscar, 1998; Bruce, 1996).

The strains of *C. jejuni* we recovered were able to persist from the farm, through transport, and into the processing plant. Although the chill tank may be a point of cross-contamination (Jones *et al.*, 1991) our study indicated that the chilling process was effective in reducing the number of positive carcasses. Cross-contamination between and within batches of broilers in the plant is not unusual (Rivoal *et al.*, 1999). Cross-contamination and colonization within the processing plant by a single clone of *Campylobacter* has been responsible for outbreaks of human disease stemming from consumption of poultry products (Allenberger *et al.*, 2003).

The core isolates were distributed in clusters randomly among several environmental samples over several flocks which also indicates a common and persistent source. A definitive common reservoir was not apparent over the course of four flocks.

Similar results were found by Peterson and Wedderkopp (2001) in that certain strains were capable of persisting in consecutive flocks. Given that *Campylobacter* is intolerant to desiccation, it is unlikely that litter is the reservoir between flocks unless the water activity was to exceed 0.85 (Shane and Stern, 2003). Darkling beetles and their larvae can act as a reservoir for *Campylobacter* (Dawe, 2005) during serial flocks. Although our research did not recover *Campylobacter* from darkling beetles, studies have indicated that *Campylobacter* strains isolated from broilers can match those recovered from darkling beetles on the farm (Jacobs-Reitsma *et al.*, 1995; Bates *et al.*, 2003).

Seasonal variations in *Campylobacter* recovery is well documented (Refregier-Petton *et al.*, 2001; Willis and Murray, 1997; Kapperud *et al.*, 1993; Jacobs-Reistma *et al.*, 1994; Wallace *et al.*, 1997). Most peaks in cecal carriage of the pathogen occurs during the warm months of summer and fall (Kapperud *et al.*, 1993; Jacobs-Reistma *et al.*, 1994; Genigeorgis *et al.*, 1986; Wallace *et al.*, 1997). Broiler carcasses in retail markets showed an increase in *Campylobacter* incidence during warmer months (Willis and Murray, 1997) while, conversely, a study of turkey carcasses indicated a surge in *Campylobacter* recovery during colder months (Logue *et al.*, 2003). A slight seasonal effect was indicated in our study when the majority of our *Campylobacter* isolates were recovered during flocks one and two. Flocks one and two corresponded with summer/fall and fall/winter, respectively. Flocks three and four were sampled during winter/spring and spring/summer seasons, respectively.

Salmonella

It was proposed by Lahellec *et al.* (1986) that the resident bacteria in the poultry house environment were the most important source of infection on chick placement day.

Our research indicated that the dominant *Salmonella* strain was capable of remaining with broilers throughout the growout and processing. Additionally, our research corroborates work by Byrd *et al.* (1999) who also found *S.* Kentucky and *S.* Heidelberg to be a problem on several breeder operations and subsequently in hatcheries.

Salmonella Kentucky and *S.* Heidelberg are common serotypes in poultry production in the United States, and are detected in the poultry house environment (Hird *et al.*, 1993; Byrd *et al.*, 1999; Roy *et al.*, 2002). The frequency at which we recovered the single *S.* Kentucky strain on both farms during multiple flocks indicates that a common source may be the cause of broiler colonization (Byrd *et al.*, 1999). Additionally, during riboprint analysis, the *S.* Kentucky strain formed a singular cluster, indicating a clonal population which further verified that isolates were from a common source (Norton *et al.*, 2001). Automated ribotyping of *Salmonella* samples recovered from the poultry house environment and processing plant has been successful (Oscar, 1998). The longitudinal nature of this study, combined with multiple sampling methods, indicates the benefit of automated ribotyping to aid broiler companies in the identification of *Salmonella* reservoirs (Bruce, 1996). In this study, the poultry house environment remained free of *S.* Kentucky during the first two flocks, but chick mortality and eggshells from the hatchery were positive. Furthermore, chick mortality, eggshells, or both were positive for *S.* Kentucky during chick placement day for all four flocks. We concluded that the hatchery or breeding stock was likely the source although neither of these locations were tested. Byrd *et al.* (1998) showed that only 5% of seeder chicks were needed to cause >50% cecal colonization of chickens.

Poultry may become infected with *Salmonella* through oral, cloacal, naval, eye, and nasal infection (Cox *et al.*, 1996). During hatching, several of these routes of infection come into play if infected eggs are placed in the hatcher. Cason *et al.* (1994) found that 86% of fertile, *S.* Typhimurium inoculated eggs hatched, and caused an increase in the number of positive hatcher air samples. Also, an uninoculated group of control eggs placed in the same hatcher yielded 44% of chicks with *Salmonella* in the intestinal tract. Hatchery debris that comes in contact with infected chicks can also become a source of contamination in the hatchery (*op. cit.*). Amendments to cleaning and disinfection regimens within a hatchery can reduce the risk of infection to chicks (Davies and Wray, 1994).

The infection status of breeder stock is reflected in the progeny so it benefits the producer to maintain healthy breeder stocks. Hatcheries may pool eggs from several breeder farms into one large-scale incubator, thereby making it difficult to identify infected breeder flocks (Chriel *et al.*, 1999). Infected parent flocks increase the risk of *S.* Typhimurium in broiler flocks and antibiotics for chicks will not be effective because horizontal transmission within the flock likely occurs before chicks arrive at the farm (*ibid.*).

Lahellec and Colin (1985) found that 1.9% of *Salmonella* strains from the hatchery remained on the carcass and that 5.8% of the hatch strain remained on the carcass to the final stage of processing. Conversely, several poultry researchers have found *Salmonella* strains that remain in flocks through to the final product (Bhatia and McNabb, 1980; Rigby and Petit, 1980; Cason *et al.*, 1994). Our *S.* Kentucky strain remained in the poultry house environment as well as cecal droppings for the duration of

each flock. The strain was also recovered from transport crate drag swabs and carcass rinses in the processing plant. Carcasses from the pre-chill tank station provided *S.* Kentucky isolates, and in one instance, a carcass provided isolates after passing through an antimicrobial dip. The potential exists for this serotype of *Salmonella* to remain on the final product and reach the consumer possibly causing infection if improperly handled or undercooked.

Lahellec and Colin (1985) recovered additional serotypes after transport and processing. It is well known that the prevalence of pathogens can increase after the stress of transport (Stern *et al.*, 1995). Also there are several points of cross-contamination in processing plants such as the scald tank, feather picking machine (Wempe *et al*., 1983) and chill tank (James *et al.*, 1992a; Lillard. 1990) where additional serotypes may be added to the carcass. *S.* Heidleberg was identified in a large number of poultry house samples but was not recovered from the transport crates or processing plant, hence this serotype was not as persistent in the broilers despite recovery from cecal droppings. Our study did not indicate the addition of new serotypes, or strains of *S.* Kentucky, following transportation or processing, but it must also be considered that our study included only one company while Lahellec and Colin (1985) reviewed several. The processing plant may simply be incapable at this time of completely eliminating *S.* Kentucky from infected incoming flocks.

This longitudinal study has created a database indicating the relationship of *C. jejuni* and *Salmonella* serotypes from day of hatch through to the final product. Long-lived strains and their reservoirs, for both *C. jejuni* and *Salmonella*, were elucidated over the course of four consecutive flocks. The reservoir for *S.* Kentucky pointed to necessary

changes in the hatchery or broiler breeder management program. Conversely, the short-lived strains may have been more susceptible to sanitation procedures. The short-lived or sporadic strains were identified with the understanding that the continuation of this project may shed more light upon their patterns of recovery. The use of the RiboPrinter® in this longitudinal study may help in developing intervention strategies for an integrated broiler operation.

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Table 1. Samples of the farm environment, transport crates, and processing plant yielding *Campylobacter* and *Salmonella* isolates from four broiler flocks.

Sample	*Campylobacter*	*Salmonella*
Water		+
Feed		+
Feed Equipment	+	+
Drag Swab	+	+
Surgical Shoe Cover	+	+
Ceca	+	+
Chick Mortality		+
Eggshells		+
Insects		+
Litter		+
Standing Water		+
Transport Crates	+	+
Pre-chill tank	+	+
Post-chill tank	+	
Post-antimicrobial Dip		+
Feed Mill		+

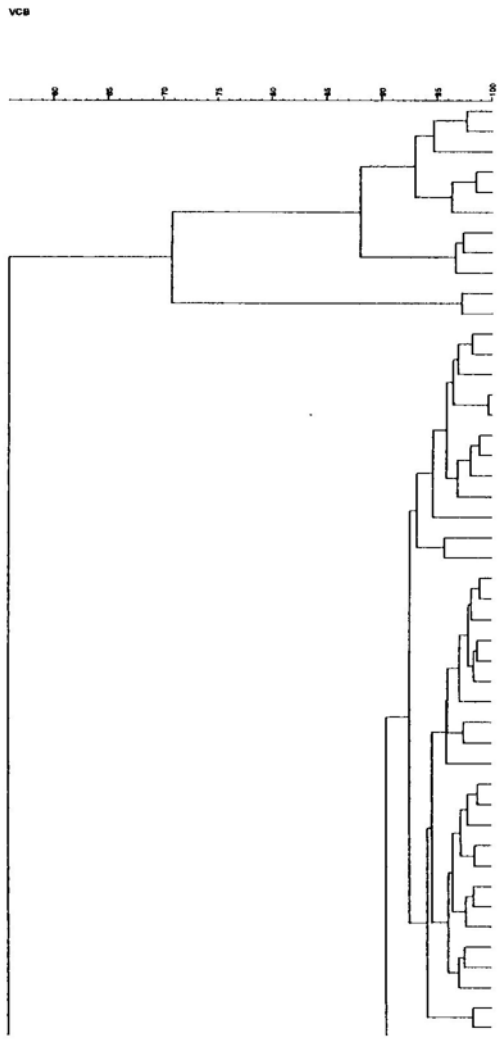
Table 2. Distribution of the three core *Campylobacter jejuni* isolates among samples collected at the farm and processing plant.

	Strain Identification N (%)		
Sample	2050	1141	1146
Cecal Droppings	16 (26.2%)	8 (27.6%)	3 (30.0%)
Surgical Shoe Covers	12 (19.7%)	4 (13.8%)	4 (40.0%)
Drag Swabs	14 (23.0%)	4 (13.8%)	1 (10.0%)
Feed Equipment	1 (1.6%)	1 (3.4%)	1 (10.0%)
Transport Crates	6 (9.8%)	5 (17.2%)	0 (0.0%)
Pre-chill tank	12 (19.7%)	7 (24.2%)	1 (10.0%)
Total	61 (100.0%)	29 (100.0%)	10 (100.0%)

Table 3. Distribution of *Salmonella* serotypes among 145 samples collected at the farm and processing plant.

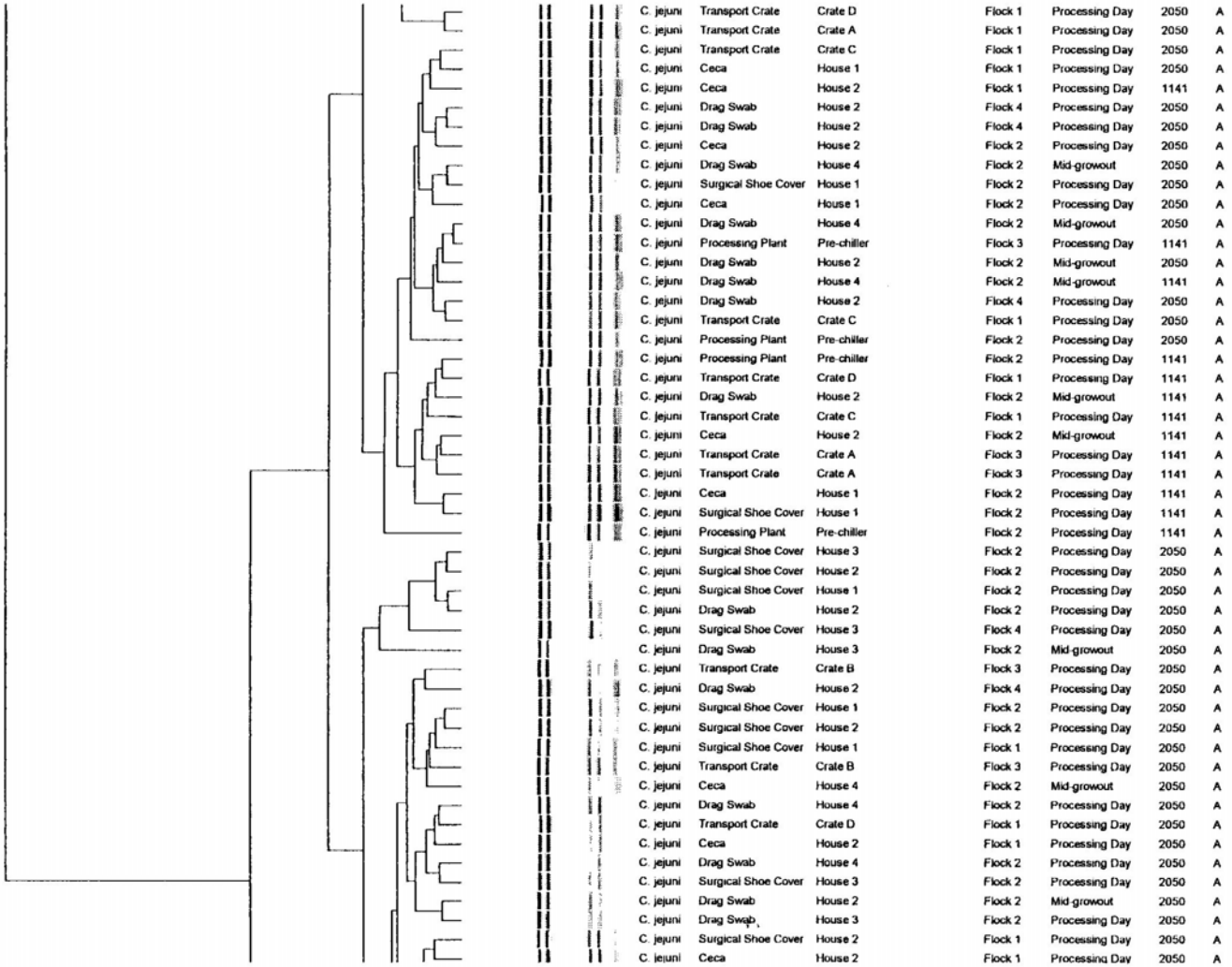
Sample	*S.* Kentucky	*S.* Heidelberg			*S.* Meleagridis	*S.* Tennessee	*S.* Hadar
		3177	3178	1069			
Surgical Shoe Covers	9 (7.7%)	9 (52.9%)	1 (50.0%)	1 (50.0%)	4 (100.0%)	1 (100.0%)	1 (100.0%)
Cecal Droppings	18 (15.5%)	1 (5.9%)	1 (50.0%)	-	-	-	-
Insects	13 (11.2%)	7 (41.2%)	-	1 (50.0%)	-	-	-
Chick Mortality	9 (7.8%)	-	-	-	-	-	-
Eggshells	10 (8.6%)	-	-	-	-	-	-
Litter	13 (11.2%)	-	-	-	-	-	-
Water	1 (0.9%)	-	-	-	-	-	-
Standing Water	2 (1.7%)	-	-	-	-	-	-
Drag Swabs	1 (0.9%)	-	-	-	-	-	-
Feed Equipment	1 (0.9%)	-	-	-	-	-	-
Feed	3 (2.6%)	-	-	-	-	-	-
Transport Crates	16 (13.8%)	-	-	-	-	-	-
Pre-chill tank	16 (13.8%)	-	-	-	-	-	-
Post-antimicrobial	4 (3.4%)	-	-	-	-	-	-
Total	116	17	2	2	4	1	1

Figure 1a. *Campylobacter jejuni* dendrogram of all isolates recovered from four consecutive broiler flocks.



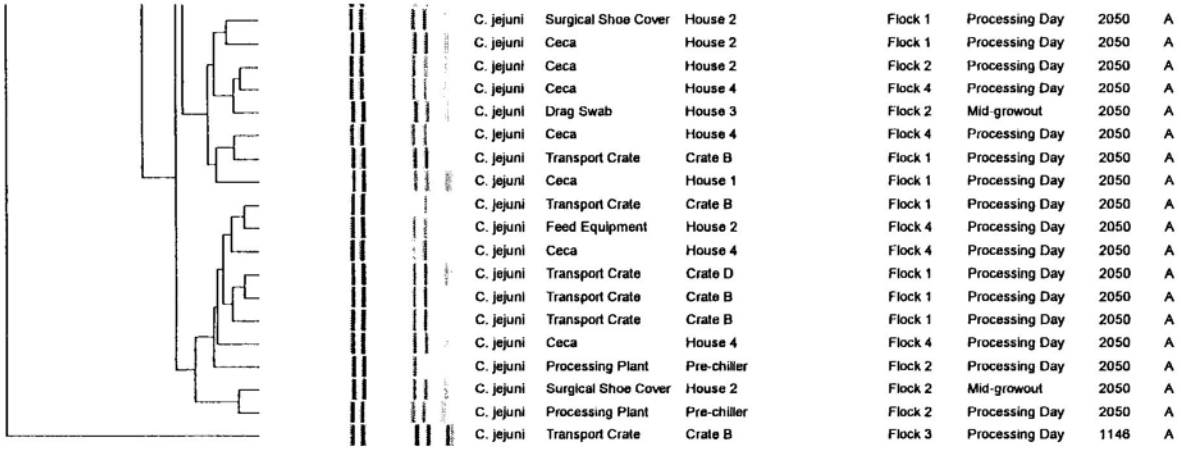
C. jejuni	Processing Plant	Post-chiller, Pre-antimicrobial	Flock 3	Processing Day	2062	A
C. jejuni	Ceca	House 1	Flock 4	Processing Day	1113	A
C. jejuni	Ceca	House 1	Flock 4	Processing Day	1112	A
C. jejuni	Ceca	House 3	Flock 4	Processing Day	2062	A
C. jejuni	Ceca	House 1	Flock 4	Processing Day	2062	A
C. jejuni	Ceca	House 1	Flock 4	Processing Day	2062	A
C. jejuni	Ceca	House 3	Flock 4	Processing Day	1165	A
C. jejuni	Ceca	House 3	Flock 4	Processing Day	1165	A
C. jejuni	Ceca	House 3	Flock 4	Processing Day	1165	A
C. jejuni	Ceca	House 2	Flock 1	Mid-growout	2072	B
C. jejuni	Ceca	House 2	Flock 1	Mid-growout	2084	B
C. jejuni	Surgical Shoe Cover	House 3	Flock 2	Mid-growout	1141	A
C. jejuni	Drag Swab	House 2	Flock 2	Mid-growout	1141	A
C. jejuni	Surgical Shoe Cover	House 3	Flock 2	Mid-growout	1141	A
C. jejuni	Ceca	House 3	Flock 2	Mid-growout	1141	A
C. jejuni	Ceca	House 3	Flock 2	Mid-growout	1141	A
C. jejuni	Surgical Shoe Cover	House 4	Flock 2	Mid-growout	1146	A
C. jejuni	Surgical Shoe Cover	House 4	Flock 2	Mid-growout	1146	A
C. jejuni	Ceca	House 2	Flock 2	Mid-growout	1146	A
C. jejuni	Feed Equipment	House 2	Flock 4	Processing Day	1146	A
C. jejuni	Processing Plant	Pre-chiller	Flock 3	Processing Day	1141	A
C. jejuni	Surgical Shoe Cover	House 4	Flock 2	Mid-growout	1146	A
C. jejuni	Feed Equipment	House 3	Flock 4	Processing Day	1141	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	2050	A
C. jejuni	Ceca	House 3	Flock 2	Mid-growout	2050	A
C. jejuni	Ceca	House 4	Flock 2	Mid-growout	2050	A
C. jejuni	Ceca	House 2	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 2	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 2	Flock 2	Mid-growout	1146	A
C. jejuni	Drag Swab	House 3	Flock 2	Mid-growout	1146	A
C. jejuni	Surgical Shoe Cover	House 4	Flock 2	Mid-growout	1146	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	2050	A
C. jejuni	Ceca	House 1	Flock 2	Processing Day	2050	A
C. jejuni	Transport Crate	Crate C	Flock 1	Processing Day	1141	A
C. jejuni	Ceca	House 4	Flock 2	Mid-growout	1146	A
C. jejuni	Ceca	House 4	Flock 2	Mid-growout	1141	A
C. jejuni	Drag Swab	House 4	Flock 2	Mid-growout	1141	A
C. jejuni	Surgical Shoe Cover	House 2	Flock 2	Mid-growout	1141	A
C. jejuni	Ceca	House 2	Flock 2	Mid-growout	1141	A
C. jejuni	Transport Crate	Crate A	Flock 3	Processing Day	1141	A
C. jejuni	Ceca	House 2	Flock 2	Mid-growout	1141	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	1141	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	2050	A
C. jejuni	Transport Crate	Crate A	Flock 1	Processing Day	1141	A
C. jejuni	Transport Crate	Crate D	Flock 1	Processing Day	2050	A
C. jejuni	Transport Crate	Crate A	Flock 1	Processing Day	2050	A

Figure 1b. *Campylobacter jejuni* dendrogram of all isolates recovered from four consecutive broiler flocks (continued).



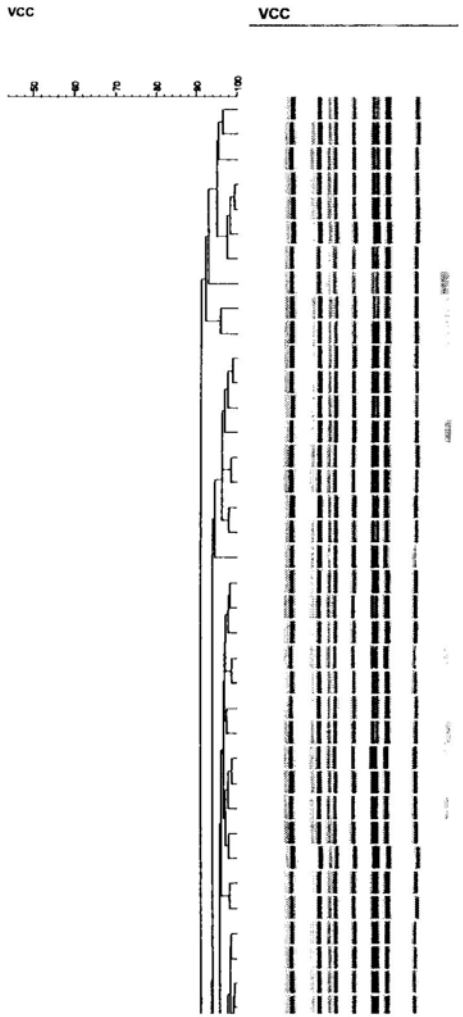
C. jejuni	Transport Crate	Crate D	Flock 1	Processing Day	2050	A
C. jejuni	Transport Crate	Crate A	Flock 1	Processing Day	2050	A
C. jejuni	Transport Crate	Crate C	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 1	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 2	Flock 1	Processing Day	1141	A
C. jejuni	Drag Swab	House 2	Flock 4	Processing Day	2050	A
C. jejuni	Drag Swab	House 2	Flock 4	Processing Day	2050	A
C. jejuni	Ceca	House 2	Flock 2	Processing Day	2050	A
C. jejuni	Drag Swab	House 4	Flock 2	Mid-growout	2050	A
C. jejuni	Surgical Shoe Cover	House 1	Flock 2	Processing Day	2050	A
C. jejuni	Ceca	House 1	Flock 2	Processing Day	2050	A
C. jejuni	Drag Swab	House 4	Flock 2	Mid-growout	2050	A
C. jejuni	Processing Plant	Pre-chiller	Flock 3	Processing Day	1141	A
C. jejuni	Drag Swab	House 2	Flock 2	Mid-growout	2050	A
C. jejuni	Drag Swab	House 4	Flock 2	Mid-growout	1141	A
C. jejuni	Drag Swab	House 2	Flock 4	Processing Day	2050	A
C. jejuni	Transport Crate	Crate C	Flock 1	Processing Day	2050	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	2050	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	1141	A
C. jejuni	Transport Crate	Crate D	Flock 1	Processing Day	1141	A
C. jejuni	Drag Swab	House 2	Flock 2	Mid-growout	1141	A
C. jejuni	Transport Crate	Crate C	Flock 1	Processing Day	1141	A
C. jejuni	Ceca	House 2	Flock 2	Mid-growout	1141	A
C. jejuni	Transport Crate	Crate A	Flock 3	Processing Day	1141	A
C. jejuni	Transport Crate	Crate A	Flock 3	Processing Day	1141	A
C. jejuni	Ceca	House 1	Flock 2	Processing Day	1141	A
C. jejuni	Surgical Shoe Cover	House 1	Flock 2	Processing Day	1141	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	1141	A
C. jejuni	Surgical Shoe Cover	House 3	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 2	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 1	Flock 2	Processing Day	2050	A
C. jejuni	Drag Swab	House 2	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 3	Flock 4	Processing Day	2050	A
C. jejuni	Drag Swab	House 3	Flock 2	Mid-growout	2050	A
C. jejuni	Transport Crate	Crate B	Flock 3	Processing Day	2050	A
C. jejuni	Drag Swab	House 2	Flock 4	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 1	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 2	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 1	Flock 1	Processing Day	2050	A
C. jejuni	Transport Crate	Crate B	Flock 3	Processing Day	2050	A
C. jejuni	Ceca	House 4	Flock 2	Mid-growout	2050	A
C. jejuni	Drag Swab	House 4	Flock 2	Processing Day	2050	A
C. jejuni	Transport Crate	Crate D	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 2	Flock 1	Processing Day	2050	A
C. jejuni	Drag Swab	House 4	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 3	Flock 2	Processing Day	2050	A
C. jejuni	Drag Swab	House 2	Flock 2	Mid-growout	2050	A
C. jejuni	Drag Swab	House 3	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 2	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 2	Flock 1	Processing Day	2050	A

Figure 1c. *Campylobacter jejuni* dendrogram of all isolates recovered from four consecutive broiler flocks (continued).



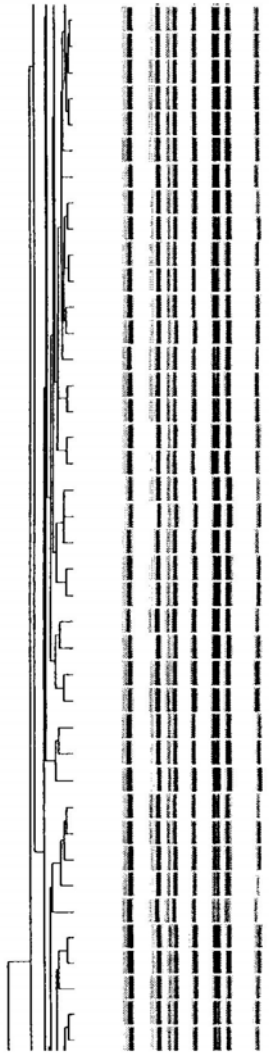
C. jejuni	Surgical Shoe Cover	House 2	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 2	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 2	Flock 2	Processing Day	2050	A
C. jejuni	Ceca	House 4	Flock 4	Processing Day	2050	A
C. jejuni	Drag Swab	House 3	Flock 2	Mid-growout	2050	A
C. jejuni	Ceca	House 4	Flock 4	Processing Day	2050	A
C. jejuni	Transport Crate	Crate B	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 1	Flock 1	Processing Day	2050	A
C. jejuni	Transport Crate	Crate B	Flock 1	Processing Day	2050	A
C. jejuni	Feed Equipment	House 2	Flock 4	Processing Day	2050	A
C. jejuni	Ceca	House 4	Flock 4	Processing Day	2050	A
C. jejuni	Transport Crate	Crate D	Flock 1	Processing Day	2050	A
C. jejuni	Transport Crate	Crate B	Flock 1	Processing Day	2050	A
C. jejuni	Transport Crate	Crate B	Flock 1	Processing Day	2050	A
C. jejuni	Ceca	House 4	Flock 4	Processing Day	2050	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	2050	A
C. jejuni	Surgical Shoe Cover	House 2	Flock 2	Mid-growout	2050	A
C. jejuni	Processing Plant	Pre-chiller	Flock 2	Processing Day	2050	A
C. jejuni	Transport Crate	Crate B	Flock 3	Processing Day	1146	A

Figure 2a. *Salmonella* spp. dendrogram of all isolates recovered from four consecutive broiler flocks.



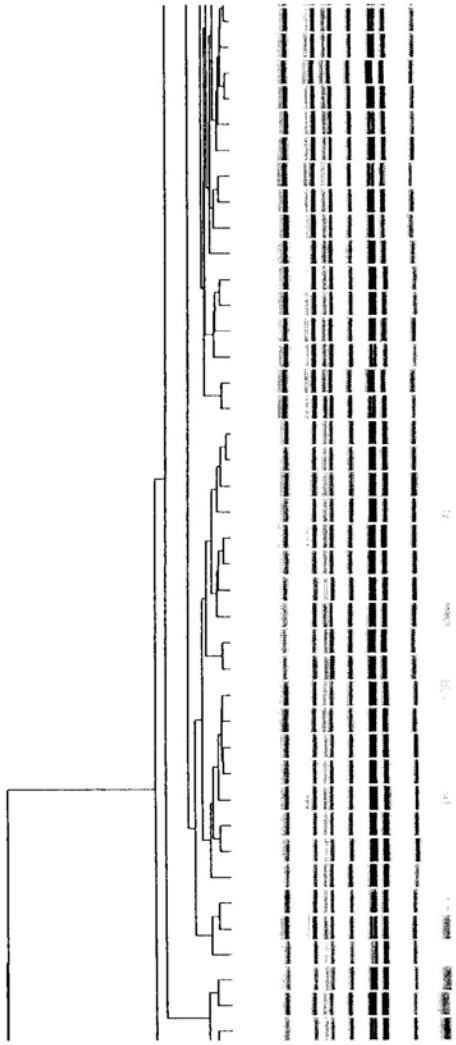
S. Kentucky	Flock 4	Chick Mortality	House 2	Chick Placement	1079
S. Kentucky	Flock 4	Insects	House 4	Processing Day	1079
S. Kentucky	Flock 3	Standing Water	House 4	Chick Placement	1079
S. Kentucky	Flock 2	Transport Crate	Crate D	Processing Day	1079
S. Kentucky	Flock 2	Transport Crate	Crate D	Processing Day	1079
S. Kentucky	Flock 2	Surgical Shoe Covers	House 4	Mid-growout	1079
S. Kentucky	Flock 2	Transport Crate	Crate C	Processing Day	1079
S. Kentucky	Flock 2	Surgical Shoe Covers	House 4	Processing Day	1079
S. Kentucky	Flock 2	Ceca	House 4	Processing Day	1079
S. Kentucky	Flock 2	Ceca	House 4	Processing Day	1079
S. Kentucky	Flock 3	Shallow Litter	House 3	Mid-growout	1079
S. Kentucky	Flock 3	Shallow Litter	House 3	Mid-growout	1079
S. Kentucky	Flock 2	Chick Mortality	House 1	Chick Placement	1079
S. Kentucky	Flock 2	Ceca	House 4	Mid-growout	1079
S. Kentucky	Flock 3	Shallow Litter	House 3	Mid-growout	1079
S. Kentucky	Flock 3	Shallow Litter	House 3	Mid-growout	1079
S. Kentucky	Flock 2	Ceca	House 4	Processing Day	1079
S. Kentucky	Flock 2	Ceca	House 4	Mid-growout	1079
S. Kentucky	Flock 2	Chick Mortality	House 1	Chick Placement	1079
S. Kentucky	Flock 1	Transport Crate	Crate B	Processing Day	1079
S. Kentucky	Flock 2	Transport Crate	Crate D	Processing Day	1079
S. Kentucky	Flock 1	Transport Crate	Crate A	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Litter	House 4	Processing Day	1079
S. Kentucky	Flock 1	Post-antimicrobial	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Litter	House 1	Mid-growout	1079
S. Kentucky	Flock 1	Water	House 2	Processing Day	1079
S. Kentucky	Flock 2	Transport Crate	Crate C	Processing Day	1079
S. Kentucky	Flock 1	Litter	House 1	Mid-growout	1079
S. Kentucky	Flock 2	Chick Mortality	House 2	Chick Placement	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 4	Insects	House 4	Processing Day	1079
S. Kentucky	Flock 4	Insects	House 4	Processing Day	1079
S. Kentucky	Flock 1	Post-antimicrobial	Processing Plant	Processing Day	1079
S. Kentucky	Flock 2	Transport Crate	Crate A	Processing Day	1079
S. Kentucky	Flock 2	Litter	House 2	Mid-growout	1079
S. Kentucky	Flock 2	Litter	House 2	Mid-growout	1079

Figure 2b. *Salmonella* spp. dendrogram of all isolates recovered from four consecutive broiler flocks (continued).



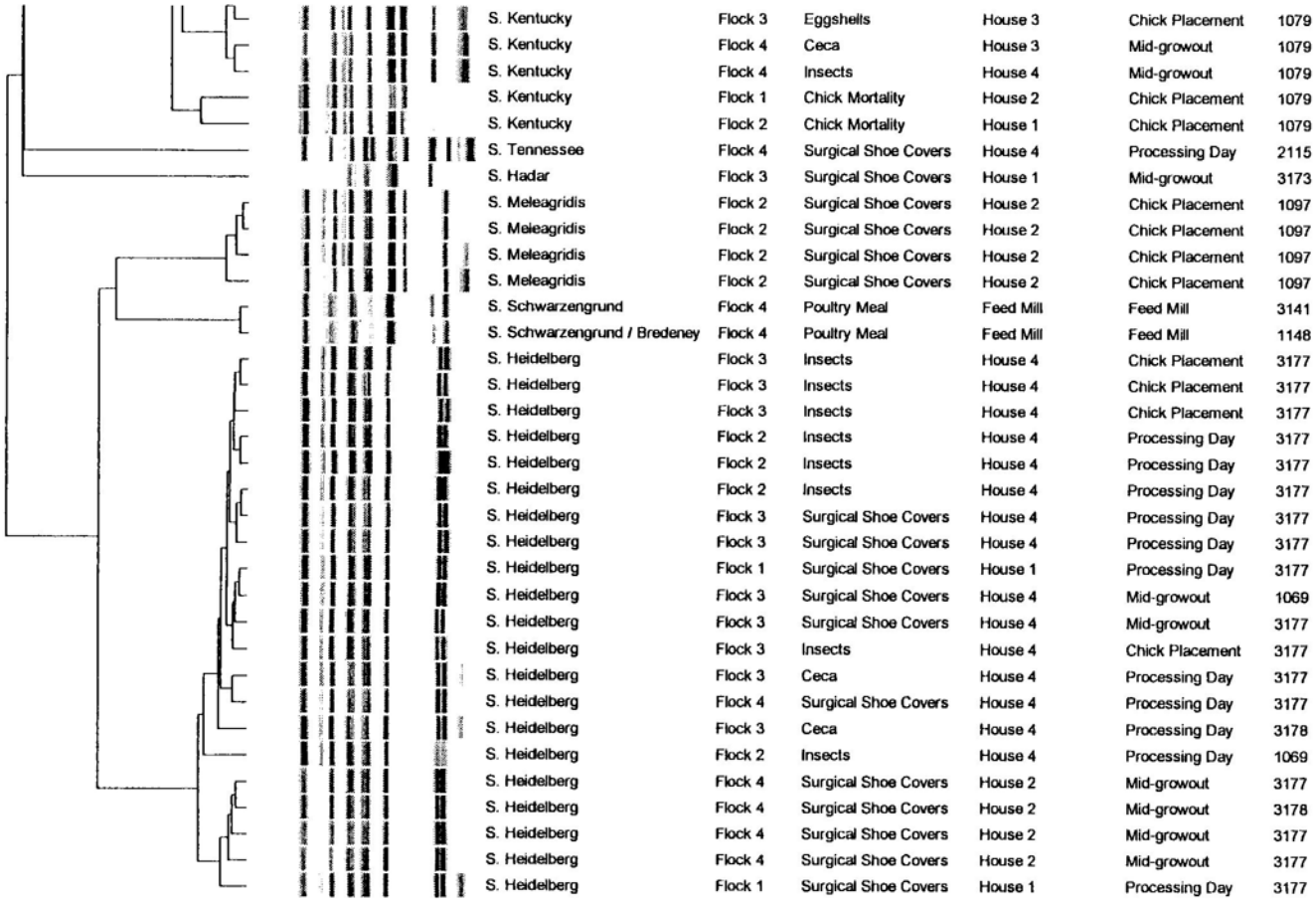
S. Kentucky	Flock 2	Litter	House 2	Mid-growout	1079
S. Kentucky	Flock 2	Litter	House 2	Mid-growout	1079
S. Kentucky	Flock 3	Insects	House 4	Processing Day	1079
S. Kentucky	Flock 1	Litter	House 4	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 2	Feed	House 3	Processing Day	1079
S. Kentucky	Flock 1	Transport Crate	Crate B	Processing Day	1079
S. Kentucky	Flock 2	Surgical Shoe Covers	House 1	Mid-growout	1079
S. Kentucky	Flock 3	Insects	House 4	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Litter	House 4	Mid-growout	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 2	Surgical Shoe Covers	House 2	Mid-growout	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 2	Surgical Shoe Covers	House 2	Mid-growout	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Ceca	House 3	Processing Day	1079
S. Kentucky	Flock 2	Surgical Shoe Covers	House 2	Mid-growout	1079
S. Kentucky	Flock 3	Insects	House 3	Processing Day	1079
S. Kentucky	Flock 3	Eggshells	House 3	Chick Placement	1079
S. Kentucky	Flock 2	Surgical Shoe Covers	House 1	Mid-growout	1079
S. Kentucky	Flock 4	Ceca	House 3	Mid-growout	1079
S. Kentucky	Flock 4	Insects	House 4	Mid-growout	1079
S. Kentucky	Flock 4	Insects	House 4	Mid-growout	1079
S. Kentucky	Flock 1	Ceca	House 3	Processing Day	1079
S. Kentucky	Flock 1	Litter	House 1	Mid-growout	1079
S. Kentucky	Flock 1	Transport Crate	Crate A	Processing Day	1079
S. Kentucky	Flock 2	Eggshells	House 1	Chick Placement	1079
S. Kentucky	Flock 3	Transport Crate	Crate A	Processing Day	1079
S. Kentucky	Flock 3	Surgical Shoe Covers	House 3	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 2	Eggshells	House 1	Chick Placement	1079
S. Kentucky	Flock 1	Chick Mortality	House 1	Chick Placement	1079
S. Kentucky	Flock 1	Chick Mortality	House 2	Chick Placement	1079
S. Kentucky	Flock 4	Insects	House 4	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079

Figure 2c. *Salmonella* spp. dendrogram of all isolates recovered from four consecutive broiler flocks (continued).



S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 3	Insects	House 4	Processing Day	1079
S. Kentucky	Flock 1	Eggshells	House 2	Chick Placement	1079
S. Kentucky	Flock 2	Insects	House 2	Mid-growout	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 2	Eggshells	House 1	Chick Placement	1079
S. Kentucky	Flock 3	Insects	House 4	Processing Day	1079
S. Kentucky	Flock 4	Ceca	House 2	Processing Day	1079
S. Kentucky	Flock 4	Ceca	House 3	Mid-growout	1079
S. Kentucky	Flock 1	Post-antimicrobial	Processing Plant	Processing Day	1079
S. Kentucky	Flock 3	Transport Crate	Crate A	Processing Day	1079
S. Kentucky	Flock 3	Standing Water	House 3	Chick Placement	1079
S. Kentucky	Flock 1	Post-antimicrobial	Processing Plant	Processing Day	1079
S. Kentucky	Flock 2	Transport Crate	Crate B	Processing Day	1079
S. Kentucky	Flock 2	Transport Crate	Crate A	Processing Day	1079
S. Kentucky	Flock 2	Feed	House 4	Processing Day	1079
S. Kentucky	Flock 1	Chick Mortality	House 1	Chick Placement	1079
S. Kentucky	Flock 2	Feed	House 4	Processing Day	1079
S. Kentucky	Flock 2	Surgical Shoe Covers	House 1	Mid-growout	1079
S. Kentucky	Flock 4	Ceca	House 4	Processing Day	1079
S. Kentucky	Flock 4	Ceca	House 4	Processing Day	1079
S. Kentucky	Flock 2	Transport Crate	Crate B	Processing Day	1079
S. Kentucky	Flock 4	Insects	House 4	Mid-growout	1079
S. Kentucky	Flock 4	Ceca	House 4	Processing Day	1079
S. Kentucky	Flock 4	Ceca	House 4	Processing Day	1079
S. Kentucky	Flock 1	Eggshells	House 1	Chick Placement	1079
S. Kentucky	Flock 1	Litter	House 1	Mid-growout	1079
S. Kentucky	Flock 1	Feed Equipment	House 1	Mid-growout	1079
S. Kentucky	Flock 1	Eggshells	House 1	Chick Placement	1079
S. Kentucky	Flock 1	Eggshells	House 2	Chick Placement	1079
S. Kentucky	Flock 2	Eggshells	House 1	Chick Placement	1079
S. Kentucky	Flock 2	Drag Swab	House 1	Mid-growout	1079
S. Kentucky	Flock 4	Ceca	House 2	Processing Day	1079
S. Kentucky	Flock 1	Ceca	House 3	Processing Day	1079
S. Kentucky	Flock 1	Pre-chiller	Processing Plant	Processing Day	1079
S. Kentucky	Flock 2	Transport Crate	Crate B	Processing Day	1079
S. Kentucky	Flock 4	Ceca	House 2	Processing Day	1079
S. Kentucky	Flock 3	Eggshells	House 3	Chick Placement	1079
S. Kentucky	Flock 4	Ceca	House 3	Mid-growout	1079

Figure 2d. *Salmonella* spp. dendrogram of all isolates recovered from four consecutive broiler flocks (continued).



S. Kentucky	Flock 3	Eggshells	House 3	Chick Placement	1079
S. Kentucky	Flock 4	Ceca	House 3	Mid-growout	1079
S. Kentucky	Flock 4	Insects	House 4	Mid-growout	1079
S. Kentucky	Flock 1	Chick Mortality	House 2	Chick Placement	1079
S. Kentucky	Flock 2	Chick Mortality	House 1	Chick Placement	1079
S. Tennessee	Flock 4	Surgical Shoe Covers	House 4	Processing Day	2115
S. Hadar	Flock 3	Surgical Shoe Covers	House 1	Mid-growout	3173
S. Meleagridis	Flock 2	Surgical Shoe Covers	House 2	Chick Placement	1097
S. Meleagridis	Flock 2	Surgical Shoe Covers	House 2	Chick Placement	1097
S. Meleagridis	Flock 2	Surgical Shoe Covers	House 2	Chick Placement	1097
S. Meleagridis	Flock 2	Surgical Shoe Covers	House 2	Chick Placement	1097
S. Schwarzengrund	Flock 4	Poultry Meal	Feed Mill	Feed Mill	3141
S. Schwarzengrund / Bredeney	Flock 4	Poultry Meal	Feed Mill	Feed Mill	1148
S. Heidelberg	Flock 3	Insects	House 4	Chick Placement	3177
S. Heidelberg	Flock 3	Insects	House 4	Chick Placement	3177
S. Heidelberg	Flock 3	Insects	House 4	Chick Placement	3177
S. Heidelberg	Flock 2	Insects	House 4	Processing Day	3177
S. Heidelberg	Flock 2	Insects	House 4	Processing Day	3177
S. Heidelberg	Flock 2	Insects	House 4	Processing Day	3177
S. Heidelberg	Flock 3	Surgical Shoe Covers	House 4	Processing Day	3177
S. Heidelberg	Flock 3	Surgical Shoe Covers	House 4	Processing Day	3177
S. Heidelberg	Flock 1	Surgical Shoe Covers	House 1	Processing Day	3177
S. Heidelberg	Flock 3	Surgical Shoe Covers	House 4	Mid-growout	1069
S. Heidelberg	Flock 3	Surgical Shoe Covers	House 4	Mid-growout	3177
S. Heidelberg	Flock 3	Insects	House 4	Chick Placement	3177
S. Heidelberg	Flock 3	Ceca	House 4	Processing Day	3177
S. Heidelberg	Flock 4	Surgical Shoe Covers	House 4	Processing Day	3177
S. Heidelberg	Flock 3	Ceca	House 4	Processing Day	3178
S. Heidelberg	Flock 2	Insects	House 4	Processing Day	1069
S. Heidelberg	Flock 4	Surgical Shoe Covers	House 2	Mid-growout	3177
S. Heidelberg	Flock 4	Surgical Shoe Covers	House 2	Mid-growout	3178
S. Heidelberg	Flock 4	Surgical Shoe Covers	House 2	Mid-growout	3177
S. Heidelberg	Flock 4	Surgical Shoe Covers	House 2	Mid-growout	3177
S. Heidelberg	Flock 1	Surgical Shoe Covers	House 1	Processing Day	3177

VI. SUMMARY AND CONCLUSIONS

As it stands, *Campylobacter* and *Salmonella* are the two food-borne pathogens that are of greatest concern to public health and well-being, and thus poultry producers. Given the current shift toward pre-harvest food safety, identification of reservoirs of *Campylobacter* on the farm is important. Much is already known about the behavior of *E. coli* and *Salmonella* on the farm and in flocks but further research must be conducted to analyze and understand the microbiology of the system as a whole. Future regulations may impose *Campylobacter* testing or screening of poultry products destined for consumers, hence the concern regarding high levels of contamination.

The improvements seen in the surgical shoe cover litter sampling indicate that the method is more effective in flock microbial testing. The recovery of *Salmonella* was higher using surgical shoe covers and they were easier to prepare and use. The recovery of *E. coli* isolates from litter samples was similar using surgical shoe covers, deep, and shallow litter samples. This indicates that the surgical shoe covers detect bacteria that are represented from all layers of litter. These results points to the fact that chickens are very active in the poultry house and can turn the litter often. *S.* Kentucky was recovered from samples of eggshells and chick mortality emanating from the hatchery. The poultry house environment from both farms did not yield *S.* Kentucky before chicks were placed until later flocks. The *S.* Kentucky that was recovered were of the same strain and formed their own cluster in the dendrogram. This clonal population of *S.* Kentucky

identified by automated ribotyping indicated a common source in the hatchery or on the breeder farms. The single strain of *S.* Kentucky also persisted from the farm and into the processing plant to the final product which may point to colonization of the plant environment or continuous cross-contamination of infected flocks. *Campylobacter* jejuni was also isolated from inside the processing plant which indicated that certain strains were more persistent that others. *Campylobacter* isolates produced three common isolates from multiple samples on a single farm but was never recovered before mid-growout sample collections. Although no definitive source was found on the farm, cecal droppings were more effective in recovering the organism.

During the course of this research several new questions were generated and further research may address these topics: 1) Given the diversity of environmental samples emanating from the poultry house, what *Campylobacter* media is best for each sample type? 2) Will isolates from the different media used to recover *Campylobacter* in the house environment differ based on the media causing certain isolates to be overlooked? 3) Would pulsed-field gel electrophoresis be able to segregate different strains from the single strain of *S*. Kentucky identified by automated ribotyping? 4) Would different styles or compositions of surgical shoe covers be more effective in *Salmonella* and *Campylobacter* recovery? 5) What distance or patterns in the poultry house should be walked to achieve maximum recovery using surgical shoe covers?

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