RECOVERY AND PERSISTENCE OF *SALMONELLA TYPHIMURIUM* AND *CAMPYLOBACTER COLI* IN YOUNG CHICKS

by

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(Under the Direction of Jeanna L. Wilson)

ABSTRACT

This research is an attempt to detect low levels of *Salmonella* Typhimurium (10^1) and *Campylobacter coli* (10^2) in broiler chicks using cloacal swabs. In these trials day old broiler chicks were orally gavaged with varying concentrations of nalidixic acid resistant *Salmonella* and gentamicin resistant *Campylobacter coli* inoculum. Deep and shallow cloacal swabs were evaluated as a non-destructive tool to identify positive *Campylobacter* and *Salmonella* broiler chicks. Along with the cloacal swabs from each chick, the spleen and liver/gall bladder were collected to determine translocation of the organisms. These studies suggest that even at low levels, *Salmonella* (10^1) and *Campylobacter* (10^2) can be recovered by cloacal swabs to help identify and control the presence of these organisms in young chicks. The deep swab detected slight more positive birds than the shallow swab. This technique has the potential be used as a non-destructive screening tool in the more valuable breeder chickens.

INDEX WORDS: *Salmonella, Campylobacter*, cloacal swabs, freezing

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DEDICATION

This thesis is dedicated in honor of my dad, Eula Lee “Butch” McLendon, thank you for teaching me what it means to be a leader and for always believing in me. This is also in honor of Dr. Scott M. Russell, your passion for microbiology and to make a difference will never be forgotten. To my daughter, Matti Lynn Tynes, what a blessing you are to me.

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CHAPTER 1

INTRODUCTION

Salmonella and *Campylobacter* are the leading cause of bacterial induced diarrheal disease in the United States and worldwide, and poultry is a significant source of these organisms. The annual cost associated with campylobacterosis in the U.S. is estimated to be $2.4 billion (Batz et al., 2012; CDC, 2011) and over 1.7 million poultry related foodborne illnesses occur annually, with over 600,000 related to salmonellosis (Hoffman et al., 2012). *Campylobacter* and *Salmonella* rarely cause fatalities in animals or humans, but they are still leading causes of foodborne diseases. Nausea, vomiting, diarrhea, fever and headaches are common symptoms of infection from these foodborne enteropathogens. Other complications have been associated with severe cases of infection including; Guillain-Barre Syndrome caused by *Campylobacter*, Reiter's Syndrome and death caused by both *salmonella* and *campylobacter* pathogens.

In 2011 the U.S passed the Food Safety Modernization Act (FSMA). This emphasized prevention rather than reacting to outbreaks as they are reported. Live poultry grow out facilities need to be an area of concentration to meet FSMA standards. The emerging Pathogens Institute concluded in 2011 that an average of approximately 1.5 million infections were caused by the consumption of poultry contaminated with *Salmonella* and/or *Campylobacter* pathogens yearly (Batz et al., 2011). Controlling contamination can be difficult due to natural infections of both *Salmonella* and *Campylobacter* in poultry by means of the oral route. Following ingestion, *Salmonella*

and *Campylobacter* invade and colonize the intestinal tract (Brownell et al., 1969; Galan and Curtiss, 1989). Once intestinal colonization has occurred, both *Salmonella* and *Campylobacter* can be shed in the feces, thus providing potential for the bacteria to spread within the flock. The spread of bacteria within a flock could increases risk to consumers. Identification of an infected flock needs to occur as quickly as possible so precautions can be taken to reduce spreading and contaminating other flocks.

A sampling tool to help evaluate flocks is needed as well as a method to improve the recovery methods of both organisms. This need is becoming more important as regulations increase and bird health, as well as performance, is becoming even more important for the poultry industry. In 2011, USDA- FSIS implemented in poultry processing plants that only 5 out of 51 sample sets could be positive for *Salmonella* and only 8 out of 51 samples positive for *Campylobacter*. These standards are likely to become a zero-tolerance level in the near future. This will be difficult since small numbers of positives can quickly turn into an entire positive flock. Poultry processing plants currently use multiple physical interventions to remove these organisms from the carcass. These steps include scalders to help remove feathers, inside – outside bird wash/rinses, antimicrobial rinses and dips and chilled storage. These steps reduce contamination, but yet consumers are still dealing with foodborne illnesses at elevated levels. Therefore, research on how these organisms thrive in live poultry flocks need further investigating. Cost effective intervention at the farm level is needed to better evaluate, control and manage these organisms. Currently flocks are evaluated by drag swabs for the environment which doesn't identify weather the birds were positive or environmental factors contributed. Birds are sampled for cecal contents which means

birds are euthanized for evaluation. These birds will never be processed for broiler meat, reducing returns for the poultry industry.

These studies evaluated the reliability of cloacal swabs to recover both *Salmonella* Typhimurium (ST). and *Campylobacter coli* (CC) in broiler chicks in a noninvasive process. This work included a total of five trials of day old broiler chicks in isolator units were orally gavaged with various inoculum levels of either (ST) or (CC). Ten samples were collected at 7, 14-21 days of age from each group to identify the lowest reliable recovery. Before euthanizing each bird was sampled by a deep and a shallow cloacal swab, proceeded by collecting samples from liver and gallbladder, spleen and ceca. The samples were taking to laboratory where samples were processed, incubated and evaluated. Colonization factors were recorded for cecal content, the (CF) value is the mean log count per gram of cecal material in each sample. Results compared cecal colonization factor (CF) to cloacal swabs as well as recovery from the liver gallbladder and spleen organs were identified. During this study two trials were also evaluated by freezing the samples at -20 C and evaluating after 14days.The results from these trials suggest cloacal swabs deep or shallow is a reliable noninvasive sampling tool for the poultry industry.

CHAPTER 2

LITERATURE REVIEW

Campylobacter

Campylobacter is a Gram-negative microaerophilic, slender (0.2-0.9 μm wide by 0.2-5.0 μm long), non-spore forming, non-fermentative bacteria. It is curved with a spiral shape, but as cells are damaged or aged a more round coccoidal appearance forms. *Campylobacter* are motile with a single flagellum at one or both poles of the cell. The cell has an inner bipolar lipid cell membrane (Humphrey et al., 2007). The outer membrane layer consists of proteins which are exposed to surface areas and promote invasion of immune responses. *Campylobacter* can grow at temperatures ranging from 30 and 46°C and pathogenic species grow optimally at 42°C (Park, 2002).

Campylobacter was originally identified in cattle as *Vibrio fetus* (King, 1957) and was correctly recognized as a human pathogen (Dekeyser et al., 1972). The use of selective enrichments and media to identify different strains has improved slightly through the years and *Campylobacter* has since become the most common cause of bacterial food-borne diarrheal worldwide (Park, 2002; Butzler, 2004; Humphrey et al., 2007). The four thermophilic *Campylobacter*s, *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*, are most often associated with human illness (McClure and Blackburn, 2004; Snelling et al., 2005). The genus *Campylobacter* belongs to the family Campylobacteraceae and there are 18 species within the genus (Humphrey et al., 2007). *Campylobacter* spp. are able to reduce nitrates to nitrite, but lack the ability to ferment

carbohydrates (McClure and Blackburn, 2004). Most infections are caused by *C. jejuni* and *C. coli*; however, *C. upsaliensis* has become an important pathogen in developing country (Humphrey et al., 2007). *Campylobacter* spp. also cause disease by infection and most individuals with campylobacteriosis develop acute gastroenteritis within 12 to 72 hours of ingestion.

Although the mechanism by which campylobacters induce disease is not well understood (Nachamkin, 2002; Snelling et al., 2005), it has been suggested that the bacteria disrupt epithelial cell function by invading and colonizing intestinal mucosa or by adhering to intestinal surfaces and producing toxins (Ketley, 1997; Park, 2002). Scientific discovery of *Campylobacter* was first found by Butzler et al., 1973) and *C. jejuni* was found in poultry by Skirrow, 1977. The bacteria were also found in drinking water (Skirrow et al., 1982) as well as in dairy milk (Jones et al. 1981). *Campylobacter* can survive in water for weeks, but is never found without fecal material present (Knill et al, 1982). Interestingly, animals are the main reservoir of the organism. It was also discovered that waterfowl and wild birds are the main natural reservoir of *Campylobacter* spps. (Luechtefeld et al., 1980). Lynch et al., 2011, detected *C. concisus* and *C. upsaliensis* at a low prevalence suggesting chickens are reservoirs for *Campylobacte*r spp. Even though these organisms are present, efforts to diminish the presence of *Campylobacter* on processed poultry have been limited in success primarily because there is a paucity of conclusive information concerning how *Campylobacter* gets into the commercial poultry flocks. This has a lot to do with the fragility of the organism and the many difficulties associated with culturing the microbe.

As regulations increase in processing facilities for *Campylobacter*, there is a need to understand how *Campylobacter* lives within broilers and how to accurately detect the levels present. This research evaluates cloacal swab techniques for recovering both *Salmonella Typhimurium* and *Campylobacter coli* in broiler birds. Improvements in sampling procedures are becoming more important to the poultry industry as federal regulations and consumer preference increase demands for *Salmonella* free products and tighter regulations on *Campylobacter* are being enforced.

Campylobacter infections in humans primarily result from direct contact with infected animals (Jones et al., 1981), consuming undercooked contaminated poultry products, or consuming other food products that were erroneously cross-contaminated during preparation (Tauxe 1992). Chickshank et al. (1982) and Humphrey et al. (2007) reported that when broiler meat consumption increased so did the incidence of campylobacterosis. Modernization of evisceration contributed to this as water and fecal material are both present during the process (Leuchtefeld and Wang, 1981). Bereswill (2011) found that a human diet with altered intestinal microbiota increases presence of E. *coli* and *Eubacterium* spp. and decreases enterococcus and *Lactobacillus* spp., which is associated with an increased susceptibility to C. *jejuni*. Therefore, *Campylobacter* infections can be caused by food contamination, direct contact with infected animals, water and on rare occasions by person to person contact.

Interagency Food Safety Analytics Collaboration (IFSAC; 2015) indicates that about 14 cases of campylobacterosis are diagnosed each year for each 100,000 persons in the population. Many other cases go undiagnosed or unreported, and campylobacteriosis is estimated to affect over 1.3 million persons every year. Campylobacteriosis occurs

more frequently in the summer months than in the winter months primarily because warmer temperatures promote growth for this organism. Another contributor possible is more consumers grill outdoors during warmer weather, increasing chances of undercooked food.

Symptoms and signs of campylobacterosis are often not distinctive enough to differentiate from those caused by other enteric pathogens. *Campylobacter* enteritis is usually self-limiting and improvements occur over several days (Ketley, 1997; Park, 2002). The minimal infection-causing dose of C. *jejuni* in humans is still unclear, but illnesses have occurred with cell counts as low as 800. Symptoms from gastrointestinal distress including diarrhea, abdominal pain, fever, nausea and vomiting are common (Humphrey et al, 2007). Acheson et al. (2001) discussed that secondary complications of *Campylobacter* infections occur as a result of direct spread from the gastrointestinal tract and can include cholecystitis, pancreatitis, peritonitis, and massive gastrointestinal hemorrhage. Extra intestinal manifestations of *Campylobacter* infection are rare and may include meningitis, endocarditis, septic arthritis, osteomyelitis, and neonatal sepsis. On rare occasions, patients infected with *C. jejuni* develop reactive arthritis, bacteremia, Guillain-Barre syndrome (GBS) (Nachamkin, 2002). Most recover within a week, though children and older adults often have complications.

Currently more than 50% of poultry sold in the United States is contaminated with *C. jejuni*. Most human infections occur by consumption of contaminated poultry and meat products but other sources include non-chlorinated water and pets as carriers. *Campylobacter* can be found on fresh processed poultry and consuming raw or under cooked chicken increases the risk of human infection (Skirrow, 1991; Altekruse et al.,

1999; Humphrey et al., 2007). Although there is potential for all commercial poultry species to become infected with *Campylobacter*, the risk is greatest for the broiler industry because of the large quantities of broiler meat consumed each year (Humphrey et al., 2007).

While vertical transmission of *Campylobacter* has not been universally accepted, several studies do substantiate the possibility. Cox et al. (2002) recovered *C. jejuni* isolates from fertile eggs of breeder hens and their progeny, suggesting that breeder hens are a source of *Campylobacter* infection. Additionally, Byrd et al. (2007) isolated *Campylobacter* from commercial hatchery tray liners, and Idris et al. (2006) detected *C. coli* in ileal, cecal, and yolk content of day old chicks, although the bacteria may have been on the surface of the eggs at the time of hatching (Newell et al., 2011). Internal organs including liver/gallbladder, spleen, thymus, ceca, and reproductive tract of broiler chicks were found to be reservoirs for *Campylobacter* (Cox et al., 2009). *Campylobacter spp* has also been isolated from the ovarian follicles (Cox et al., 2005) and reproductive tracts (Camarda et al., 2000; Buhr et al., 2002; Cox et al., 2009) of laying and broiler breeder hens, therefore strongly suggesting that fertile egg contents can become contaminated during egg formation.

This implies that breeder hens are a source of *Campylobacter* contamination, and these results further support the possibility of transmission. Cox et al. (2002) recovered *C. jejuni* isolates from breeder hens and their progeny that had identical ribotype patterns and *fla*A short variable region DNA sequences.

Although studies have shown *Campylobacter* spp. can colonize internal and reproductive orgins of laying hens (Camarda et al., 2000; Cox et al., 2009), table eggs

have not been identified as a significant source of *Campylobacter* infection in humans thus far. Studies have shown that the prevalence of *Campylobacter* among table eggs is low (Doyle, 1984; Izat and Gardner, 1988; Adesiyun et al., 2005; Sulonen et al., 2007). The growth characteristics (thermophilic and microaerophilic) of *Campylobacter* place severe restrictions on its ability to survive outside of the host (Park, 2002; McClure and Blackburn, 2004), and unlike *Salmonella* spp., *Campylobacter* spp. are generally not capable of multiplying in foods during processing or storage (Park, 2002). Reducing contamination at the farm level could influence the presence of campylobacter in processing facilities. Newell et al (2011) suggests that a 2.0 $\log^{10}$ reduction of *Campylobacter* cells per broiler carcass would result in a 30-fold decrease in human campylobacterosis.

Prevention and Control Campylobacter

Control of outbreaks are greatly dependent upon transmission of the organism from farm animals such as poultry and dairy cows, as well as other factors such as pasteurization of milk (Gill, Bates and Lander, 1981: Waterman, 1982), purification of drinking water, following Good Manufacturing Practices (GMPs) in processing facilities, good hygiene while working with animals, and hygienic practices in the kitchen. Studies show that mishandling and/or consuming contaminated and undercooked poultry increases chances of campylobacterosis greatly (Tauxe, 1992).

Over the last decade, research efforts have focused on recovering *Campylobacter* from poultry, but most efforts to reduce the contamination have been at the processing plant not at the farm. Research on environmental influences, such as housing equipment

including; nests, feeders and drinker lines in broiler breeder laying houses, have extensively been focused on *Salmonella* control. Very little research has been done to identify sources of *Campylobacter* at the farm level (Newell et al., 2003). It is apparent that primarily controlling *Campylobacter* in poultry flocks is more difficult than controlling *Salmonella.* This is primarily due to the fact that *Campylobacter's* natural growth environment (most optimal growth temperature) is the same as internal body temperature of chickens. Therefore, biosecurity on the farms is critical in controlling human infections from the organism. More research needs to be performed on interventions at the farm level in reducing *Campylobacter* in poultry flocks.

Farm management practices impact the control of *Campylobacter* several different ways. One is water supply management. Unchlorinated water can be a source for *Campylobacter. Campylobacter* can be ingested and colonize within the digestive tract of the birds (Pearson et al., 1993). The risk for infected flocks increases with the number of houses on each farm, even when all in-all out practices are used. When there are three or more houses on a farm, biosecurity becomes highly important as chances for contamination increases (Refregier-Petton et al., 2001; McDowell et al., 2008; Bouwknegt et al., 2004). A major concern is human traffic. Increased number of people entering a farm biosecurity area increases the risk of a breach. Humphrey (2006) reported that thinning out flocks doubles *Campylobacter* infection rates as this required personnel to handle the entire flock. There is a direct correlation of the number of farm helpers, the number of visits and the number of positive *Campylobacter* flocks (Hauneau-Salaun et al., 2007; Refregier- Petton et al., 2001). Other sources of flock contamination include catch crews, farms close by livestock, and other farm animals nearby (Hutchison et al.,

2004). The presence of other animals near poultry housing could also influence *Campylobacter* in poultry flocks. Various types of domestic livestock and wild animals, such as birds, cattle, deer, dogs, pigs, and sheep, are natural reservoirs for the zoonotic pathogen, *Campylobacter* (Humphrey et al., 2007). The presence of these animals and surrounding poultry could influence horizontal transmission of *Campylobacter* from an environmental source for flock infection. Rodents, insects, contaminated water supply, and poor facility maintenance (Newell and Fearnley, 2003) are additional factors for increased introduction of *Campylobacter*.

Commercial poultry have been identified as a significant reservoir for *Campylobacter* infection in humans as *C. jejuni*, the species most often associated with Campylobacteriosis, can easily colonize in the avian digestive tract (Snelling et al., 2005; Pope et al., 2007) and area prevalent organism in poultry farm environments (Camarda et al., 2000; Newell and Fearnley, 2003). When infection has occurred, *Campylobacter* can spread rapidly throughout the flock. This increases the chances of positive carcasses at the processing facilities. Molecular techniques suggest that up to 80% of human infections are transmitted from poultry to humans (European Food Safety Authority, 2010). This puts more pressure on the poultry industry to reduce risks of contamination starting at the farm level before the organism ever reaches the processing plant.

Salmonella

Salmonella spp. are small (0.7-1.5 μm by 2 to 5 μm), rod shaped, Gram-negative, anaerobic bacteria that are generally motile with peritrichous flagella (Bell, 2004). The optimum growth temperature for *Salmonella* spp. is 37°C (Holt et al., 1994). It was first

identified in 1884 when *Salmonella choleraesuis* was isolated from swine with cholera. Since then, *Salmonella* has been identified as a human pathogen causing foodborne illnesses.

Salmonella belong to the family Enterobacteriaceae and can be differentiated from other bacteria in the family by the utilization of thiosulfate with hydrogen sulfide production, and the inability to ferment lactose (Cox et al., 2000). There are two species (*Salmonella* bongori and *Salmonella* enterica) and over 2500 serovars of S. *enterica*. S. *Enteritidis* became the top serotype isolated from infections in the United States in 1990 (Mishu et al., 1994).

In 1996, the United States developed Foodborne Disease Active Surveillance Network (FoodNet), a system to correlate lab results and track the bacterial infections. Hazard Analysis and Control Points (HACCP) became mandatory in food (meat and poultry) processing plants in 1997. These systems together help positively identify pathogens and control foodborne pathogens to help reduce outbreaks. *Salmonella* is currently estimated to be responsible for over 1 million illnesses yearly (CDC, 2011). The number of *Salmonella* positive samples allowed in processing facilities has decreased about 20% (FSIS, 2012). Over 1.5 million poultry related foodborne illnesses occur annually, with over 600,000 related to salmonellosis (Batz et al., 2011). This has the poultry industry focused on preventive measures to control and manage *Salmonella* at the farm level before the birds arrive at the processing plants.

The infection route of *S. Enteritidis* to humans begins with environmental contamination of the housing facility. Flies (Olsen and Hammock, 2000; Holt et al., 2007), rodents (Garber et al., 2003; Meerburg and Kijlstra, 2007), humans (Guard-Petter,

2001), and wild birds (Craven et al., 2000) have all been identified as possible sources for *Salmonella* transmission. Once inside the housing facility, *S. Enteritidis* must adapt to and multiply within the environment. It has been suggested that the survival of *Salmonella* through the food chain is partially due to its ability to respond effectively to environmental changes for survival (Humphrey, 2004). *Salmonella* infection is usually self–limiting, lasts for a week, and causes diarrhea, fever and nausea within 1 to 3 days. In rare cases, Reiter's Syndrome and death have been associated with salmonellosis. This usually occurs in immune compromised individuals, infants, and the elderly (Thomson et al., 1995).

Factors Affecting Salmonella Colonization

Following colonization, *S. Enteritidis* can be shed through the feces and eventually spread throughout the flocks. Risk factors associated with the horizontal spread of *S. Enteritidis* include housing system, large flock size, and airborne transmission (Mollenhorst et al., 2005). Hens subjected to feed withdrawal are more susceptible to *S. Enteritidis* infection (Holt, 1993, 1995; Ricke, 2003; Dunkley et al., 2007).

Over the past 20 years, *S. Enteritidis* has been the leading cause of salmonellosis worldwide, both *S. Typhimurium* and *S. Enteritidis* are most commonly associated with human salmonellosis in the U.S. (CDC, 2006). Milner and Shaffer (1952) inoculated day old chicks with 10 cells and Cox et al. (1990) found that intracloacally inoculated day-old chicks could be colonized with as few as two cells of *Salmonella*, which was 100 times fewer than the number required to colonize older chicks. Chickens generally become

more resistant to *Salmonella* colonization with age and the establishment of intestinal microflora (Hannah et al., 2010).

Numerous factors are involved in determining how susceptible chickens are to intestinal colonization with *Salmonella* spp. (Bailey, 1987). An important factor is the age of the chicken. Newly hatched chicks are thought to be most susceptible to *Salmonella* colonization because they lack a fully mature gut microflora in the intestinal tract during the first two weeks after hatch (Cox et al., 1996). The number of cells required for colonization is dependent upon the pathogen's ability to survive passage through the gastrointestinal tract. Natural infection frequently occurs following ingestion. *Salmonella* first encounter the acidic (pH ~4.5-5) environment of the crop. *Lactobacillus* present in the crop creates this low pH, but upon feed withdrawal, the lactobacilli population decreases and crop pH increases (Durant et al., 1999). As a result, the crop becomes a more suitable environment for the bacteria to survive and colonize. The proventriculus and gizzard are also acidic environments in which *Salmonella* must survive. In an *in vitro* study, Cox et al. (1972) decreased survivability of *Salmonella* spp. at a pH of 2.0 corresponding to the proventriculus and gizzard. On the other hand, because the pH of the small intestine and large intestine is not as acidic this allows more growth to occur. Research has suggested that birds subjected to feed withdrawal are more susceptible to *Salmonella* colonization (Holt, 1993, 1995; Durant et al., 1999; Ricke, 2003; Dunkley et al., 2007). This is a common practice in the broiler industry.

Intestinal colonization can be affected by the dose level and strain of *Salmonella* chickens are subjected to (Bailey, 1987) and the ability of *Salmonella* to attach, colonize, and invade intestinal tissues (D'Aoust et al., 1991). Other factors known to affect

Salmonella colonization include environmental and physiological stresses, health status, medication effects, and diet (Bailey, 1987; 1993). Bacterial colonization and invasion are influenced by environmental stimuli (Dunkley et al., 2008). Scientists continue to research serovar and host specific colonization factors and mechanisms to further the understanding of *Salmonella* in poultry flocks. *S. enterica* is a significant pathogen in animal production and potentially impacts animal welfare. Measurements to prevent *Salmonella* colonization are important to reducing infections (Humphrey 2006).

Prevention and Control Salmonella

Even though regulations are tighter, HACCP is implemented, and FoodNet has increased requirements, *Salmonella* outbreaks continue to be a problem for the poultry industry and consumers. *Salmonella* prevention at the farm level is similar to *Campylobacte*r prevention. Rodent control is a priority in reducing *Salmonella* at grow-out facilities. The pathogen can easily be carried from the outside to the inside of a house and infect an entire flock by spreading contaminated fecal droppings (Davies et al., 1995). Beetle control, along with house fly control are also important.). Beetles introduced to the grow- out facility increase introduction of bacteria to the environment. These can contaminate the water supply and reduce litter quality (Olsen et al., 2003. Disinfecting housing in between flocks is a must, as any organic material can carry the organism. Personnel biosecurity should also be a priority as well; it has been shown that 20% of worker's boots are contaminated with *Salmonella* and are potential carriers to infect flocks (Bailey et al., 2001, Bailey et al., 2007, Hendricks et al., 2007).

Probiotics, vaccinations, and antibiotics are currently being used to lower the number of *Salmonella* positive birds in poultry flocks. Maternal antibodies due to vaccination has also helped reduce numbers in progeny (Avila et al., 2006). Antibiotics on the other hand have been helpful in the past, but more drug resistant organisms are prevailing and restrictions on antibiotic use are tightening as well.

Cloacal Swabs

Cloacal swabs have been used on freshly slaughtered turkeys and fresh fecal samples on transport crates (Leuchtefeld and Wang 1981; Northcutt et al., 2003). Pearson (1993) used cloacal swabs on 230 day old chicks, but samples cultured negative for *Salmonella* and *Campylobacter*. Craven et al. (2000) used cloacal swabs on wild birds, but results were negative. Quessy and Messier, (1992) recovered *Salmonella* and *Campylobacter* from Ring- Billed Gulls using cloacal swabs with a recovery percentages 2.4-45.2% for *Campylobacter* and 4.3-29.6% for *Salmonella.* Kapperud et al., 1993 recovered *Campylobacter* on broilers at slaughter before scalding and defeathering with cloacal swabs but only 10% sampled positive. *Salmonella* Enteritidis was recovered in hens at 4% by cloacal swabs (Garcia et al., 2011). In Norway 540 cloacal swabs were taken from 40 different species of wild birds the results were C. *fetus* subsp. *jejuni* 28.4%, and *Salmonella* .8%. Even though research with cloacal swabs has been conducted on freshly slaughtered carcasses (Luechtefeld et al., 1981) to recover *Salmonella* (Lee, et al., 1980), no extensive research has been performed with cloacal swabs on live birds to recover *Campylobacter*. Shanker et al. (1988) did challenged chicks with a single 0.2 ml inoculum of *C. jejuni* into the crop or cloaca at 2 to 3 days and

2 weeks old. Cloacal swabs were taken at 1, 3, 7 and 14 days post challenge. Birds challenged at 2 to 3 days were euthanized and cultured at 14 days and the two week old birds were sacrificed at four weeks of age and sampled. Inoculum levels of 10^2, 10^4, and 10^6 were used and cloacal swabs were taken. There was 189 *C. jejuni*-positive chicks, 117 (62 %) were colonized by day 1, 167 (88 %) by day 3, and 184 (97 %) by day 7. In this study it took a minimum of 10^4 to recover *Campylobacter* from orally gavaged chicks. Depth of cloacal swabs or material was not disclosed. These data showed that to maintain a *Campylobacter* free flock up to processing age a better understanding at the farm level is essential. Luechtefeld et al., 1981 found 100% of cloacal swab samples to be positive using Campy-thio antimicrobial agent to recover organism from carcasses. Even though research has progressed for *Campylobacter* in the last decade, more sensitive recovery methods are needed to assess the prevalence of the organism in live poultry.

Laboratory

Reduced oxygen (5-10%) environment needs to be present for campylobacter cultures to grow because they are microaerophilic. *Camylobacter ssp.* optimal growth temperature is 42^{o}C. The spiral shape and grey color in appearance help to positively identify the cells under a microscope. The growth characteristics (thermophilic and microaerophilic) of *Campylobacter* place severe restrictions on its ability to survive outside of the host (Park, 2002; McClure and Blackburn, 2004).

Gentamicin resistant marker strain for C. *coli* was obtained during 2002-2005 for CDC and National Antimicrobial Resistant Monitoring System (NARMS). This marker strain allows controlled research to be performed so we have a better understanding of the

colonization of *Campylobacter* (Cox et al., 2009). Selective media allows typical colonies to be readily identified. The microaerophilic environment and the presence of gentamicin reduces and/or inhibits the growth of extraneous bacteria as well.

Frozen

Little research has been done on freezing *Campylobacter* and *Salmonella* samples. This would provide a way to store field samples at the lab until they could be processed. Sahin et al. (2003) found that *C. jejuni* could survive in egg yolk for up to 14 days when eggs were stored at below optimal growth temperatures of 18°C. C. *jejuni* has been shown to display physiological activity at 4°C **(**Hazeleger et al., 1998). *C. jejuni* does survive during frozen storage in poultry samples (Beuchat, et al., 1987; Lee et al., 1998; Solow et al., 2003). A portion of the microflora may be killed due to these factors; some cells may survive but will likely be injured. The same is true for research for recovering *Salmonella* from chickens and freezing the samples, but there is some research of frozen carcasses, frozen raw pork (Escartín et al,. 2000) and raw and par fried chicken strips and nuggets (MacDougall et al., 2004). *Salmonella* has been found on frozen carcass samples. Izat (et al., 1991) found *Salmonella* from twelve carcasses that were sampled using a three- tube most probable number technique. Recovered *Salmonella* ranged from 5 to 34 organisms per 100mL fluid rinse samples. The isolate serotypes were found to be *Salmonella typhimurium, Salmonella paratyphi,* and *Salmonella arizonae.* This is evidence that *Salmonella* can be recovered after being frozen which helps the industry be able to sample flocks and eliminate time sensitivity on analyzing fresh samples.

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CHAPTER 3

DETECTING *CAMPYLOBACTER COLI* IN YOUNG CHICKS USING TWO DIFFERENT CLOACAL SWABS TECHNIQUES[1]

[1]B.L. McLendon, N.A. Cox, D.E. Cosby, E.R. Montiel, S.M. Russell, C.L. Hofacre, M.A. Landrum, J.S. Jackson and J.L. Wilson. To be submitted to The Journal of Applied Poultry Research.

Abstract

Day old chicks (n=25) were orally gavaged with either a low inoculum 10^{1-3} or high 10^6 *Campylobacter* (Cc) gentamicin resistant marker strain. Chicks were placed in separate isolation units, and 7 to 21d post challenge 10 birds per group were subjected to a shallow (9mm) and a deep (24mm) cloacal swab. Each swab was placed into 5 mL of Tecra® broth without added supplements, vortexed, streaked for isolation onto Campy Cefex agar plus 200 ppm gentamicin, then supplements were added to each tube prior to enrichment for detection of Cc from negative swabs. After swabbing, birds were sacrificed and one cecum was quantitatively analyzed for Cc from the control group; both ceca were removed from all challenged birds and analyzed for Cc as appropriate. After 14d, 95% of the shallow and 90% of the deep swabs were positive for Cc. Even with a relatively low inoculum ($\leq 10^3$) Cc achieved a high degree of cecal colonization and the cloacal swab (either shallow or deep) proved to be a reliable indicator for Cc. Birds challenged with $>10^2$, after 7 and 14 d were colonized with $>10^6$ cells. After 7d, all shallow and deep swabs were positive for Cc regardless of challenge dose. Since it might not be practical in an industry setting to process the swabs the day of collection, we looked at the reliability of cloacal swabs after freezing for up to 21 d. When the level in the ceca was high, recovery of Cc was excellent but when the level was low ($\leq 10^2$ inoculum level), recovery was very unreliable. If the levels of *Campylobacter* are relatively high in the ceca, both the shallow and deep swabs, unfrozen or frozen, are reliable nondestructive methods to detect this microorganism.

Keywords: cloacal swab, *Campylobacter*, broiler chicks, bacteria

Introduction

Campylobacter is a commonly reported bacterium causing foodborne illnesses and has become a worldwide concern. A significant portion of human diarrhea infections are attributed to undercooked poultry and poultry products. *Campylobacter* has a natural growth temperature of 37 - 42°C, similar to the internal body temperature of live poultry, allowing the organism to become a commensal inhabitant of the intestinal tract (Oosterom et al., 1983; Humphrey, 1995). This provides a good environment for *Campylobacter* and may explain why poultry and poultry products are associated with approximately 70% of human foodborne campylobacterosis (Kornacki et al., 2003; Idris et al., 2006). *Campylobacter* spp. are commonly found in both broilers and broiler breeders. Research efforts to diminish the presence of *Campylobacter* on processed poultry have had only limited success primarily because farm intervention has been hampered by a paucity of conclusive information concerning how the organism gets into commercial poultry flocks. No extensive research using cloacal swabs to detect *Campylobacter* has been reported. Cloacal swabs of wild birds near broiler housing were taken but no positive results for *Campylobacter jejuni*, *Salmonella* spp. or *Clostridium perfringen*s were identified (Craven et al., 2000).

Intervention strategies are needed at the rearing and breeder levels as well as at the hatchery to reduce the level of *Campylobacter* spp. present on the live birds entering the processing plant, thus allowing processors to decrease the levels of *Campylobacter* on raw product leaving the processing facilities. The objective of this research was to evaluate the ability of two different cloacal swab techniques for determining, with a non-destructive method, the presence of *Campylobacter* and the effect of freezing the swabs

on the results. Both the sampling procedures and freezing option would provide the poultry industry with a non-destructive inexpensive method for detecting *Campylobacter* in expensive breeder chicks and a way to preserve the samples until laboratory analysis can be completed. Cloacal swabs would allow one to positively identify infected birds. In previous research, vent swabs on 49 d old broilers were used on freshly slaughtered broilers and only had 37% positives (Pearson et al., 1993). This research evaluates two different swab techniques, a shallow cloacal swab and a deeper colon swab on live broiler chicks. We, also, looked at preserving the samples, which would allow more time and flexibility to process the swabs. Having a tool to detect *Campylobacter* at the grandparent and parent breeder farms would allow the industry to identify if antibacterial and/or probiotic use is necessary to provide a chick with less *Campylobacter* to the grow-out facility and ultimately the processing plants. This technique would be able to help evaluate biosecurity measures in the breeder management programs.

Materials and Methods

Animal husbandry

Day old chicks were obtained from a local hatchery, transported to the University of Georgia Poultry Research Farm and housed in isolation units (IU, Controlled Isolation Systems Inc., San Diego, CA). The IUs were equipped with nipple drinkers, open feeders and wire mesh flooring. Air exchange inside the IU was provided by a filtered, positive pressure, high-efficiency particulate arrestance (HEPA) ventilation system. Chicks were fed unmedicated starter feed for the entire experiment. Twenty-five chicks were placed in each IU and orally gavaged with 0.1 mL of a low or high inoculum level (10^{1-3} or 10^{6}) of

a gentamicin resistant *Campylobacter coli* (**Cc**) marker strain (Table 3.3) (Cox el at., 2009) at placement. In an attempt to achieve varying levels of Cc in the ceca (in a second experiment), 3 seeder birds were inoculated per IU with either 10^3 or 10^6 CFU of Cc (Appendix A). Adequate brooding temperatures were maintained and birds had *ad libitum* access to feed and water on a 24 h light regimen.

Cloacal Swab Techniques

At 7 and 14 d of age, 10 birds per IU were subjected to a shallow (9 mm), followed by a deep (24 mm) cloacal swab using two sterile polyester tipped applicators (Pur-Wraps, Puritan Medical Products Co, LLC, Guilford ME). The shallow swab was inserted into the cloaca first, followed by a deep swab. Each swab was placed into separately labeled, 13x100 mm screw cap tubes containing Tecra® Broth (TB, 3M Corp., St. Paul, MN) without supplements. The tubes were vortexed then streaked for isolation onto Campy Cefex plates with 200 ppm gentamicin (CcGen) added. The plates were incubated for 48 h at 42°C in a microaerobic environment (5% O_2, 10% CO_2 and 85% N_2). Supplements were added to each tube and all tubes were incubated under microaerobic conditions overnight at 42°C. When the streaked plates were negative, the tube was vortexed and restreaked for isolation onto CcGen plates and incubated overnight at 42°C in a microaerobic environment.

Frozen

The same collection procedures used for the shallow and deep cloacal swabs were also used for the frozen samples from days 7, 10, 14 and 21, except each swab was placed into 5 mL of Tecra® broth (Frenchs Forest, NSW, Australia) without supplements and

15% glycerol was added. All tubes were frozen at -20°C and after 7, 14, or 21 d the tubes were thawed at ambient temperature for 4 h. The tubes were vortexed and the same recovery methods were used as described above.

Cecal Samples

After collection of the swab samples, the broiler chicks were euthanized by cervical dislocation. The exterior of the chick was sprayed with 70% ETOH to disinfect the surface and the skin with feathers was separated and removed from the muscle wall, which was again sprayed with 70% ETOH. The ceca were aseptically removed and placed into sterile stomacher bags (Fisher Scientific, Pittsburgh, PA). Bags were labeled accordingly, placed on ice and transported to the lab for analysis. The ceca were macerated with a rubber mallet to ensure the contents were exposed. TB was added to the ceca at a ratio of 3:1 volume to weight of the ceca before stomaching for 60 s (Technar Company, Cincinnati, OH). A semi quantitative method was used to determine the number of cells per gram in the ceca (Blanchfield et al., 1984; Cox et al., 2015). After stomaching, two cotton tipped applicators were placed into the contents of the bag. Swab one was spread plated onto a CcGen (A plate). Swab two was transferred to a tube containing 9.9 mL of TB without supplements, vortexed and a third swab was inserted into the tube, moistened and spread plated onto CcGen (B plate). The contents of the tube were poured into the stomacher bag containing the ceca and incubated at 42^{o}C under microaerobic conditions and along with the spread plates for 48 h. Any samples with negative results were restreaked from the enriched ceca onto a fresh CcGen plate and the plate incubated for 48 h at 42^{o}C under microaerobic conditions. Plate counts were

estimated to the nearest $\log_{10}$ and the CFU/g ceca was calculated and recorded. The recovery percentage data for deep and shallow swabs were analyzed using an One-way Anova with a significance of $p \leq 0.05$ (SAS JMP Pro V. 13).

Results and Discussion

The reliability of a shallow and deep swab to detect Cc in one and two-week-old chicks are shown in Table 3.1. There was no difference between the shallow and the deep swab in detecting positive samples. Each performed equally well, regardless of the age of the chick. However, the concentration of the marker strain of Cc in the ceca was critical regarding the performance of the cloacal swabs. When the level in the ceca (CFU/g of ceca and cecal material) was greater than $\log_{10}$ 6.0, the shallow swab detected the marker strain in 81/90 (90%) samples and the deep swab 84/90 (93.3%). However, when the level in the ceca was less than 10^6, both the shallow and the deep swab detected the marker *Campylobacter* only 32% of the time. So it was very reliable for high levels and somewhat questionable at detecting lower level colonization. Regardless of the level of the marker in the ceca, there was no difference in the ability of either swabbing technique to detect *Campylobacter.* We had hoped for varying levels of colonization but that did not occur. Even when we used a low inoculum level, the ceca of the inoculated birds became highly colonized. Once the organism passed the gizzard proliferation occurred and the number of organisms increased greatly no matter the level of inoculum orally gavaged. Seeder birds were placed in the pens with intention to obtain varying levels of colonization, but this was also unsuccessful because high colonization occurred in all of the pen mates of the seeder birds as well (data not presented).

Since it might not be practical in an industry setting to process swabs on the collection day, we decided to test the effects of freezing the swabs for up to 14 d at -20°C. Sahin et al. (2003) found that *C. jejuni* could survive in egg yolk for up to 14 d when eggs were stored at below optimal growth temperatures at 18°C.

Freezing did not seem to have an adverse effect, but the inoculum level once again seemed to play a critical role. The hostile acidity of the proventriculus and gizzard might be injuring or killing all of the Cc cells when the lowest inoculum levels (10^1) were used as was demonstrated by Cox et al. (1972) against *Salmonella* spp. Therefore, either no live cells or only a few injured cells reached the ceca, which did not lead to a high colonization of the ceca. When this occurs, the cloacal swab is very unreliable. However, when a 10^3 inoculum was used, some cells were able to reach the ceca, proliferate to high levels and were detected by cloacal swabbing. In Table 3.2, there were 46 chicks inoculated with 10^1 cells and only two were positive with either a shallow or a deep swab. There were 20 chicks inoculated with 10^3 cells and even after freezing the shallow swab detected *Campylobacter* in 19/20 and with the deep swab 20/20 were positive. In the unfrozen samples, similar results were observed (Table 3.2). When the level in the ceca was high, recovery of Cc was excellent but when the level was low (from 10^1 or 10^2 inoculum level), recovery was very unreliable. If the levels of *Campylobacter* are relatively high in the ceca, both the shallow and deep swabs, unfrozen or frozen are reliable nondestructive methods to detect this microorganism. It would appear from the data in this study (Tables 3.1 and 3.2), both the shallow and deep swab, either unfrozen or frozen were very reliable as long as the level of *Campylobacter* in the ceca was > 10^1/g.

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Table 3.1. Detection of CcGen in one and two-week-old chicks using a shallow and deep cloacal swab

Age at sampling	Inoculum[a,b]	Number of chicks Sampled	Number Positive		Level in Ceca[a]
			Shallow	Deep	
7 days	10^3	10	9	10	7.5
	10^6	10	9	10	7.7
	10^2	15	15	14	6.1
	10^1	10	1	2	1.5
	10^3	10	10	10	7.1
14 days	10^3	10	8	9	8.7
	10^6	10	10	9	8.7
	10^2	30	28	29	8.4
	10^1	10	0	0	1.5
	10^3	10	7	7	7.2

[a] Chicks orally gavaged with CcGen at day of age.

[b] Log value of *C. coli* per gram of ceca and cecal content at 7 and 14 days of age.

No significant differences between deep and shallow technique were noted.

Table 3.2. Effect of freezing for 14 days at -20°C on the ability of cloacal swabs (shallow or deep) to detect CcGen in young chicks

Age at sampling	Inoculum[a]	Number of chicks Sampled	Number Positive	
			Shallow	Deep
7 days	10^1	10	0	0
	10^3	10	10	10
10 days	10^1	11	2	1
14 days	10^1	10	0	0
	10^3	10	9	10
21 days	10^1	15	0	1

[a] Chicks orally gavaged with CcGen at day of age.

No significant differences between deep and shallow technique were noted

Table 3.3. Levels of inoculum gavaged were determined by a series of studies. Concentration levels of inoculum were reduced after recovery results were finalized to find the lowest level to successfully recovery *Campylobacter coli* (Cc) by cloacal swabs.

Trial	inoculum level (Cc)
Trial 1	$8.1x10^3$ $8.1x10^6$
Trail 2	$6.10x10^2$
Trial 3	$1.7x10^1$ $1.7x10^3$
Trial 4	$3.4X10^1$ $3.4X10^1$

CHAPTER 4

DETECTION OF *SALMONELLA* IN YOUNG CHICKS WITH CLOACAL SWABS[1]

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Abstract

Newly hatched broiler chicks (n=25) obtained from a local commercial hatchery were orally gavaged with $10^1 – 10^5$ cells of a nalidixic acid resistant strain of *Salmonella* Typhimurium (ST). The inoculum (0.5 mL) was gavaged into the crop using a 20-gauge animal feeding needle. After inoculation, birds were placed in isolation units containing nipple drinkers, mesh flooring and feeders. At 7 and 14 days' post challenge, 10 birds per treatment were subjected to a shallow followed by a deep cloacal swab using two sterile polyester tipped applicators. The shallow swab was inserted first approximately 2 cm into the cloaca followed by the deep swab, inserted to a depth of approximately 2 cm into the colon. Each swab was placed into separate, labeled, screw cap tubes containing 5.0 mL of 0.05% buffered peptone water (BPW). Tubes were vortex and streaked for isolation onto brilliant green sulfa agar plates with 200 ppm nalidixic acid (BGS w/Nal). The tubes and plates were incubated for 24 h at 37^{o}C. The plates were observed for typical growth and positive results recorded. Where results were negative, the pre-enriched tube was vortexed and struck for isolation onto BGS w/ Nal plates, incubated for 24 h at 37^{o}C. The procedure was repeated for the frozen samples at 7, 10, 14 or 21 days of age with the exception of the addition of 15% glycerol to the BPW and then frozen at -20^{o}C for 14 days. On d 14 of storage, the frozen swabs were thawed and analyzed as above. After cloacal swabbing, the chickens were humanely euthanized; the ceca were aseptically removed; place into a sterile stomacher bag; macerated with a rubber mallet; diluted 1:3 w/v with BPW; and stomached for 60 s. A semi-quantitative method was used to determine the approximate CFU/g of cecal material. When > 10^6 CFU/g was recovered in the cecal material for the unfrozen swabs, detection with either

method was 47/50 (97%) and when < 10^6 CFU/g was recovered in the cecal material, detection with either method fell to 38/60 (63.3%). Since there was no difference between the shallow and deep methods, the shallow method would be preferred as there is less chance of injury to the chick. After freezing the cloacal swab samples for 14 d, the shallow method detected 32/64 (50%) and the deep detected 418/34 (64%). These results indicate that if you are unable to analyze the samples on the day of collection, storing them for up to 14 d at -20^{o}C does not adversely affect recovery. The deep swab method was slightly better than the shallow when frozen. So, if unable to process the same day, the deep swab would be preferable, but if able to process the day of collection (most preferred method), the shallow swab would be preferable.

Introduction

Salmonella is one of the leading foodborne pathogens often found on poultry products. For the poultry industry, controlling and reducing the presence of this organism is a priority. Federal regulations are already in place using HACCP in order to reduce risks of *Salmonella* but in the future, meeting these standards will become even more important. Over the last decade the poultry industry has increased biosecurity at the farm level and introduced *Salmonella* vaccination programs in parents stocks (Dorea et al., 2010; Immerseel et al., 2005). Even with these interventions, a portion of the health and safety risks of salmonellosis is still connected to raw poultry products. Many factors contribute to *Salmonella* in poultry, one of which is a newly hatched chick is susceptible to *Salmonella* colonization because its intestinal microflora is still immature (Cox et al., 1996). Milner and Shaffer (1952) found day old chicks could be readily colonized with

very low doses of *Salmonella*. Another study by Cox et al., (1990) demonstrated the same results. Chickens exposed to higher doses of *Salmonella* have a higher probability of becoming colonized with some *Salmonella* spp colonizing the avian intestinal tract more efficiently than others (Barrow et al., 1988).

The objective of this research was to evaluate non- destructive methods to detect the presence of *Salmonella* in valuable breeder chicks. Many of the techniques used today, chick box liners, litter drag swabs, dust swabs, air samples and feed samples, do not directly evaluate how many individual birds are positive for *Salmonella* (Dorea et al., 2010). Cloacal swabs are a minimally invasive method which might provide reliable data which can be used to evaluate flock *Salmonella* status. Previous research using cloacal swabs has been carried out on freshly slaughtered carcasses (Luechetfeld et al., 1981). Intervention during live production, especially at the breeder flock level, is an important place to begin controlling the organism. Intervention at the breeder flock level could reduce the load of *Salmonella* being introduced to broiler farms, essentially reducing the amount of *Salmonella* present on live birds at the processing facilities. A non-destructive sampling method (shallow and/or deep cloacal swabbing) was evaluated. Since the poultry industry might not have the lab resources available for immediate evaluation of samples, freezing the swab samples was also evaluated.

Materials and Methods

Newly hatched broiler chicks were obtained from a local hatchery and transferred within an hour to the University of Georgia Poultry Research Center. Twenty-five birds were orally gavaged with an inoculum ranging from 10^1 to 10^5 (Table 4.3.) cells of a

nalidixic acid resistant strain of *Salmonella* Typhimurium (ST). The inoculum (Appendix B), 0.5 mL volume, was gavaged directly into the crop using a 20 gauge animal feeding needle (Popper and Sons, Inc., New Hyde Park, NY; Cox et al., 1990). After inoculation, birds were placed in isolation units (IU, Controlled Isolation Systems Inc., San Diego, CA). The units contained nipple drinkers, mesh flooring and feeders for the chicks. Air exchange inside the IU was provided by a filtered positive pressure HEPA ventilation system. Chicks were fed non-medicated starter feed for the length of the experiment. Adequate brooding temperatures were maintained and birds had *ad libitum* access to feed and water on a 24-hour light regimen.

Cloacal swabs (Unfrozen)

At 7 and 14 days' post challenge 10 birds per treatment were subjected to a shallow, followed by a deep cloacal swab using two sterile polyester tipped applicators (Pur-Wraps, Puritan Medical Products Co, LLC, Guilford ME). The shallow swab was inserted first at 1 cm in the cloaca followed by a deep swab inserted to a depth of 2 cm into the colon. Each swab was placed into separately labeled, 13x100 mm screw cap tubes containing 5.0 mL of 0.05% buffered peptone water (BPW). Excess length of the swab handle was cut with alcohol rinsed scissors. Samples were taken to the lab at Russell Research Center to be processed. Tubes were vortexed; streaked for isolation onto brilliant green sulfur agar (Difco/Becton, Dickinson and Co, Sparks, MD) with 200 ppm of nalidixic acid (Sigma Chemical Co., St Louis, MO; BGS w/Nal) added; and the plates and the sample tubes were incubated 24 h at 37°C. Plates were observed for typical growth and if present recorded as positive, however when the streaked plates were

negative, the incubated sample tube was vortexed and streaked for isolation onto BGS w/Nal plates and incubated 24 h at 37°C, after which final results were recorded.

Cloacal swabs (Frozen)

The same collection procedures used for the unfrozen shallow and deep cloacal swabs were used for the frozen samples from 7, 10, 14 and 21 day old chicks except that each swab was placed into 5 mL of 0.05% BPW plus 15% glycerol (Sigma, St Louis, MO). The samples were stored frozen at -20°C for fourteen days, removed from frozen storage, thawed at ambient temperature for 4 h and processed for analysis according to the same sampling procedure as the unfrozen samples. The plates and tubes were incubated for 24 h at 37°C. Positive results were recorded; negative samples were struck onto BGS w/Nal agar plates following the same protocol used for the unfrozen samples, after which final results were recorded. Random colonies from both the frozen and unfrozen samples were checked to determine that they exhibited the same biochemical reactions and serogroup as the ST used for inoculation.

Ceca sampling

After collection of the swab samples the broiler chicks were humanly euthanized and the exterior of chick was sprayed with 70% ETOH (Pharmco-Aaper, Brookfield, CT) to disinfect the surface. The skin, along with feathers, was separated and removed from the muscle wall. The muscle wall was sprayed with 70% ETOH and an incision was made with an alcohol/flamed scalpel. The ceca were aseptically removed and placed into sterile stomacher bags (Fisher Scientific, Pittsburgh, PA). Bags were labeled

accordingly, placed on ice and transported to the laboratory for analysis. The ceca were macerated using a rubber mallet to ensure the contents were exposed. BPW was added to the ceca at a ratio of 3:1 v/w before stomaching for 60 s (Technar Company, Cincinnati, OH). A semi-quantitative method was used to estimate the CFU/g in the ceca (Blanchfield et al., 1984). Briefly, two cotton tipped applicators were inserted into the bag containing the ceca/BPW solution. One swab was spread plated on BGS w/Nal plates (A plate). The second cotton tipped applicator was transferred to a tube containing 9.9 mL of BPW and vortexed for 10 s. A third cotton tipped applicator was inserted into the tube and spread plated onto a second BGS w/Nal plate (B plate). The contents of the tube were returned to the stomacher bag containing the ceca. Plates and stomacher bags were incubated at 37^{o}C for 24 h. When both spread plates were negative, a fourth cotton tipped applicator was inserted into the pre-enriched cecal samples and spread plated onto a fresh BGS w/Nal plate (C plate). The $\log_{10}$ value CFU/g of cecal material were calculated, recorded and log transformed. The recovery percentage data for deep and shallow swabs were analyzed using an One-way Anova with a significance of $p \leq 0.05$ (SAS JMP Pro V. 13).

Results and Discussion

The results of a shallow and deep swab to detect *Salmonella* in young chicks 7 or 14 days of age are shown in Table 1. When the level of colonization in the ceca was > 10^6 CFU/gm, detection by either shallow or deep swab was 47/50 (97%). When ST colonization in the ceca was < 10^6 CFU/g, the detection rate by either shallow or deep swab was 38/60 (63.3%). However, regardless of the level of ST in the ceca, there was

no difference in the detection rate between the shallow or the deep swab. Even when using lower inoculum levels, a high rate of ST colonization in the ceca was obtained, allowing both shallow and deep swabs to be almost all positive at 7 d of age. Therefore, the shallow would be the better method to use because of less risk of injury to the chick. A non-destructive tool such as a cloacal swab is very important to the poultry industry to allow the breeder companies to determine if *Salmonella* is present in the valuable breeder chicks without the necessity to sacrifice any animals.

Due to various constraints (such as sampling multiple flocks, distance between the farm and laboratory, etc.) the poultry industry may not be able to analyze the swab results on the same day as collection. Therefore, we decided to evaluate if freezing the swabs for as long as 14 days would have any adverse effect on the recovery of ST. Table4. 2 shows the detection rates after freezing the cloacal swab samples from 7 to 21 day old chicks. These day-of-hatch chicks were orally inoculated with 10^1 to 10^3 ST cells per chick. After sampling and freezing for 14 days, the shallow swab detected 32/64 (50%) while the deep swab detected 41/64 (64%). Freezing did not seem to make a difference in sensitivity. If you are unable to analyze the swabs right away and you must freeze them, it would seem that the deep colonal swab would give the best indication of *Salmonella* contamination.

The level in the ceca (for the frozen swab samples) was not determined, but from the percent positive of the frozen swabs suggests that the levels were $< 10^6$ since similar results (50-64%) were obtained with unfrozen samples when the level of ST was $< 10^6$ (Table4.1). Even after 14 days of freezing, using cloacal swabs gives a sufficient indication of *Salmonella* contamination in young chicks. So you can freeze if you must,

but doing the analysis the same day as the swabbing (unfrozen) technique seems to be slightly more reliable.

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Table 4.1. Use of a shallow and deep cloacal swab to detect *Salmonella* in young chicks

No significant differences between deep and shallow technique were noted

Number Positive					
Age of Chick	Inoculum	# Sampled of each	Shallow	Deep	Level in ceca
7d	10^5	10	10	10	$>10^6$
	10^3	10	10	10	$>10^6$
	10^2	15	14	14	$<10^6$
	10^3	10	10	9	$<10^6$
	10^1	10	8	8	$>10^6$
14d	10^5	10	9	9	$>10^6$
	10^3	10	10	10	$>10^6$
	10^3	10	6	7	$<10^6$
	10^1	10	8	9	$<10^6$

Table 4.2. Effect of freezing for 14d on the shallow and deep swab to detect *Salmonella* in young chicks

Number of Positives				
Age of Chick	Inoculum	# Sampled	Shallow	Deep
7d	10^1	10	3	6
	10^3	10	5	8
10d	10^2	11	7	9
14d	10^1	9	2	3
	10^3	10	6	7
21d	10^2	14	9	8

No significant differences between deep and shallow technique were noted

Table 4.3 Levels ST (Nal) inoculum orally gavaged

Trial	inoculum level (ST)
Trial 1	$1.54x10^5$ $1.54x10^3$
Trail 2	$2.35x10^2$
Trial 3	$3.8x10^3$ $3.8x10^1$
Trial 4	$1.8X10^2$ $1.8X10^2$

Levels of inoculum gavaged were determined by a series of studies Concentration levels of inoculum were reduced after each study after recovery results were final to find the lowest level to successfully recovery *Salmonella* Typhimurium (ST) by cloacal swabs.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Purchasing chicks (broiler and layers) are expensive and valuable to the poultry industry. Industry needs a reliable, non-destructive method to screen these birds for the presence of *Salmonella* and *Campylobacter.* These studies demonstrated that either a shallow or deep cloacal swab had no significant difference and is an effective method to detect *Salmonella* and *Campylobacter* without sacrificing or injuring the chicks. Results suggest that cloacal swabs give accurate evaluations comparable to ceca, L/GB and spleen. Freezing the swabs for analysis at a later time did not diminish the effectiveness of this method which makes it more useful to the poultry industry, as samples would need to be transported to internal or external laboratories for processing

Cloacal swabs to recovery both *Salmonella* and *Campylobacter* from birds could benefit the industry in directly identifying flock source issues and preventing these organisms from spreading. This would reduce the numbers of organisms entering the processing facilities therefore reducing chances of foodborne illnesses from poultry for consumers.

APPENDICES

The appendices are included to provide explanation of how inoculum concentrations were prepared for the trials. Appendix A describes how *Campylobacter coli* (Gentamicin resistant) inoculum was prepared. Appendix B describes how *Salmonella Typhimurium* (Naladixic resistant) inoculum was prepared.

APPENDIX A

INOCULUM CONCENTRATION (CC GEN)

A *Campylobacter coli* inoculum was prepared by spreading a stock culture on to onto Campy Cefex plates (Accumedia, Lancaster, MI) with 200 ppm gentamicin (CcGen) added. The plates were incubated for 48 h at 42°C in a microaerobic environment (5% O_2, 10% CO_2 and 85% N_2). The growing colonies of (CcGen)were transferred from the plates and serial diluted with a 0.85% saline solution until concentration of $1x10^1$ or $1x10^6$ cells/ml were reached using a Spectronic 20D+ spectrophotometer (Thermo Spectronic, Madison, WI). In these preliminary tests, chicks were challenged orally with 10^1 to 10^6 cfu/mL of CC gen. In these preliminary trials ceca were all positive at the higher doses (Table 3). In order to assure chicks with very low levels of CC the low-level inoculum was reduced to 10^1 or 10^2 cfu/mL of CC Gen.

Newly hatched broiler chicks were obtained from a local hatchery and transferred within an hour to the University of Georgia Poultry Research Center. Twenty-five birds were orally gavaged with an inoculum ranging from 10^1 to 10^5 cells of a Gentamicin resistant strain of *Campylobacter coli* (CC). The 0.5 mL of inoculum, was gavaged directly into the chick's crop using a 20-gauge animal feeding needle (Popper and Sons, Inc., New Hyde Park, NY; Cox et al., 1990). After inoculation, birds were placed in isolation units (IU, Controlled Isolation Systems Inc., SanDiego, CA). The units contained nipple drinkers, mesh flooring and feeders for the chicks. Air exchange inside the IU was provided by a filtered positive pressure HEPA ventilation system. Chicks

were fed non-medicated starter feed for the length of the experiment. Adequate brooding temperatures were maintained and birds had *ad libitum* access to feed and water on a 24-hour light regimen.

APPENDIX B

INOCULATION CONCENTRATION (NAL ST)

A nalidixic acid-resistant *Salmonella* Typhimurium (NAL ST) inoculum was prepared by spreading a stock culture on to nutrient agar (NA) plates (Accumedia, Lancaster, MI) and incubated at 37^{o}C for 24 hours. The growing colonies of NAL SE were transferred from the plates and serial diluted with a 0.85% saline solution until concentration of 1x10^{1} or 1x10^{6} cells/ml were reached using a Spectronic 20D+ spectrophotometer (Thermo Spectronic, Madison, WI). In these preliminary tests, chicks were challenged orally with 10^{1} to 10^{6} cfu/mL of Nal ST. In these preliminary trials ceca were all positive at the higher doses (Table 3). In order to assure chicks with very low levels of ST the low-level inoculum was reduced to 10^{1} or 10^{2} cfu/mL of Nal ST.

Newly hatched broiler chicks were obtained from a local hatchery and transferred within an hour to the University of Georgia Poultry Research Center. Twenty-five birds were orally gavaged with an inoculum ranging from 10^{1} to 10^{5} cells of a nalidixic acid resistant strain of *Salmonella* Typhimurium (ST). The 0.5 mL of inoculum, was gavaged directly into the chick's crop using a 20-gauge animal feeding needle (Popper and Sons, Inc., New Hyde Park, NY; Cox et al., 1990). After inoculation, birds were placed in isolation units (IU, Controlled Isolation Systems Inc., SanDiego, CA). The units contained nipple drinkers, mesh flooring and feeders for the chicks. Air exchange inside the IU was provided by a filtered positive pressure HEPA ventilation system. Chicks were fed non-medicated starter feed for the length of the experiment. Adequate brooding

temperatures were maintained and birds had *ad libitum* access to feed and water on a 24-hour light regimen.