Campylobacter jejuni and *Salmonella* spp. Detection in Chicken Grow Out Houses by Environmental Sampling Methods

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ABSTRACT

Campylobacter and *Salmonella* are foodborne pathogens commonly associated with raw poultry. Although there has been much research done on isolating these pathogens from poultry production environments using cloacal swabs, fecal samples, intestinal tract contents and dissection, research involving environmental sampling has been limited. New and/or improved environmental sampling methods may provide an easy, convenient, and less time-consuming way to collect samples. Coupling these sampling methods with PCR may provide a relatively simple, rapid, and robust means of testing for foodborne pathogens in a chicken house or flock prior to slaughter.

Air, boot and sponge samples were collected from three commercial chicken grow-out houses located in southwestern Virginia when flocks were three, four, and five weeks old. Air samples were collected onto gelatin filters. Fecal/litter samples were collected from disposable booties worn over investigator's protective shoe coverings. Pre-moistened sponges were used to sample house feed pans and water dispensers on drink lines. A PCR method

was used to qualitatively detect *Campylobacter jejuni* and *Salmonella* spp. *Campylobacter jejuni* was detected at each farm (house), across all three ages (3, 4, and 5 weeks), and from each sample type. *Salmonella* was not detected in any of the environmental samples. For all 270 samples, 41% (110/270) were positive for *Campylobacter.* Collectively, 28% (25/90) of air, 44% (40/90) of sponge, and 50% (45/90) of bootie samples were positive for *Campylobacter*. The methods used in this study are non-invasive to live animals, relatively rapid and specific, and could enable poultry processing facilities to coordinate scheduled processing of flocks with lower pathogen incidence, as a way to reduce post-slaughter pathogen transmission.

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DEDICATION

I dedicate this body of work to my parents Rita and Dan Kuntz for their love and support.

INTRODUCTION

The United States poultry industry produced 36.1 billion pounds of chicken in 2007, with Americans consuming 86 pounds per person in 2006 (ERS 2008). To handle this large demand chickens are raised in large flocks, usually numbering more than 30,000 chicks at start, in large grow-out houses. This large scale rearing makes it very easy for pathogenic bacteria to spread within a flock. While *Campylobacter* and *Salmonella* do not cause illness in birds they do have a serious impact on human health and are the most important foodborne pathogens associated with raw or undercooked poultry meat. The US Department of Agriculture (USDA) currently has regulations governing proper control of *Salmonella* within slaughter plants but there are no regulations or testing done before slaughter, at the farm level. Although *Campylobacter* is the leading cause of bacterial gastroenteritis (Moore 2005) the USDA has no mandates for its control in poultry processing and production. The USDA Food Safety and Inspection Service (FSIS) have been conducting baseline studies since 2006 to investigate the prevalence and quantitative level of *Campylobacter* on broiler carcasses within slaughter facilities (FSIS 2008a). The data collected from these studies will be used to develop standards for process control of *Campylobacter* which may become mandatory by the end of this year. The USDA does try to help farmers reduce the prevalence of *Campylobacter* and *Salmonella* within their flocks by providing voluntary guidelines for controlling these pathogens (FSIS 2008b).

The routes of transmission for which *Campylobacter* and *Salmonella* are spread throughout a flock are not fully understood. Consequently, control and eradication of these pathogens is difficult once they are present within a poultry house. Chickens are able to harbor these pathogens asymptomatically so internal organs of chickens are often tested to confirm a positive infection within a flock (Cox et al. 2007). The chickens excrete the pathogens in their stool, so it could generally be assumed a flock is infected if *Campylobacter* or *Salmonella* are found within the poultry house in fecal droppings (Stern et al. 2003). Sampling in and around the poultry environment has been conducted to try to identify reservoirs where these pathogens survive and to try to find answers as to how they are transmitted throughout entire flocks in relatively short time periods (Berndtson 1996, Devane 2005). Controlling the spread of these pathogens within flocks could help to make our food supply safer by reducing the amount of harmful bacteria going through the slaughter plants.

The purpose of this study is to add to the methods used in environmental sampling studies. By incorporating the use of air sampling, swab sampling, and fecal samples the condition of the flock can be monitored for colonization of either pathogen, *Campylobacter* or *Salmonella*, quickly and easily without the need to physically inspect any of the chickens. This could, in turn, allow processors to reduce their overall incidence of the pathogens within the slaughter plant.

CHAPTER ONE

LITERATURE REVIEW

Campylobacter

Characteristics of Organism

Campylobacter jejuni is a gram negative spiral rod in the family *Campylobacteraceae* (Montville et al. 2005). Within the *Campylobacter* genus there are fourteen or more species; the most important species to human health are two of the thermotolerant campylobacters, *C. jejuni* and *C. coli*. (Snelling et al. 2005). *C. jejuni* is estimated to be the leading cause of bacterial diarrheal illness in the United States (FDA 2007), and is associated with 85% of campylobacteriosis cases, while the rest is predominately caused by *C. coli* (Keener et al. 2004). Organisms in the *Campylobacter* genus are microaerophilic, with optimal growth conditions consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen at 42ºC (FDA 2007). *C. jejuni* cannot grow below 30˚C but has been shown to survive in temperatures as low as 4˚C (Hazeleger et al. 1998; Holler et al. 1998), and it will survive when held in material at refrigeration temperatures better than at room temperature (Montville et al. 2005). *C. jejuni* is motile, using a single flagellum at one or both ends of the cell to move in a corkscrew-like motion (Snelling et al. 2005).

. Adaptation to aerophilic growth

Although normal atmospheric levels of oxygen are toxic to *C. jejuni*, some laboratory studies have shown that it can be cultured to grow at normal

atmospheric levels of oxygen. *Campylobacter* has been observed, in vitro, to grow aerobically at cell densities higher than 10^7 CFU/ml, when they formed biofilms, but at cell densities less than 10^4 CFU/ml *Campylobacter* is less tolerant to oxygen and no aerobic growth was observed (Kaakoush et al. 2007). Chynoweth et al. (1998) also found that *Campylobacter* could be cultured to grow in aerophilic conditions, then subsequently passed multiple times under either aerobic or micro-aerobic conditions.

Role of Humidity and Water

Campylobacter is very sensitive to drying. In a study done by Line (2006), low humidity was found to affect the rate at which *Campylobacter* colonized subsequent chickens. Line found that if the relative humidity of the room was 30%, there would not be enough surviving *Campylobacter* to infect subsequent birds, even if they were placed on the same litter 24 hours after contaminated birds had been removed. But if the relative humidity was 75-85%, which is common in poultry production facilities, then chicks had to be held for at least one week after the contaminated chicks had been removed to prevent infection (Line 2006).

Prevalence and Foodborne Illness

Campylobacters are well established environmental contaminates on farms and in poultry houses, with many studies and reviews done to investigate their transmission, spread, and eventual contamination of food products (Keener et al. 2004; Hansson et al. 2007). Infection with *Campylobacter* in humans causes campylobacteriosis. The onset of this illness is usually within two to five

days of ingesting the bacterium (FDA 2007). In most cases the infection is self limiting, dissipating in seven to ten days. The most common symptoms associated with campylobacteriosis are diarrhea, sometimes bloody, fever, abdominal cramping, nausea, headache, and muscle pain (Altekruse et al. 1999). In rare cases, infections can eventually lead to reactive arthritis or a paralyzing disease known as Guillain-Barré syndrome (Leonard et al. 2004). The Centers for Disease Control estimates that about 124 deaths per year can be attributed to *Campylobacter* infections (Altekruse et al. 1999). Since infections are generally passed within ten days, antibiotics are not regularly required, but if they are, erythromycin or fluoroquinolnes are usually prescribed (Keener et al. 2004).

Approximately 2.4 million cases of campylobacteriosis occur yearly in the United States, 85% of which are caused by *C. jejuni* (Friedman et al. 2000). The infective dose is thought to be as few as 400 to 500 bacteria (FDA 2007). Allos et al. (2001) estimated that 50-70% of *Campylobacter* foodborne illness has been associated with poultry. Raw milk, un-chlorinated water, and contact with pets or farm animals have also been the source of outbreaks (Keener et al. 2004). *Campylobacter* has been found in cattle, sheep, swine, and many avian species (Keener et al. 2004).

Salmonella

Characteristics of Organism

Salmonella is a facultatively anaerobic gram-negative motile rod in the *Enterobacteriaceae* family (Montville et al. 2005). There are two species of

Salmonella, with the enteric species encompassing all disease causing *Salmonella.* Currently there are over 2,000 serotypes (Brenner et al. 2000). The most common involved in outbreaks and isolated from humans are *Salmonella enterica* serotype Enteriditis and *Salmonella enterica* serotype Typhimurium (CDC 2008). *Salmonella enterica* serotype Typhi is the bacterium responsible for typhoid fever. The optimal growth temperature of *Salmonella* is 37˚C but it has been known to grow in foods as high as 54˚C and as low as 2˚C (Montville et al. 2005). *Salmonella* is also very adaptive to pH changes, being able to grow in a pH range of 4.5 to 9.5, even though its optimal pH is 6.5 to 7.5 (Montville et al. 2005).

People infected with nontyphoid serotypes of *Salmonella*, commonly exhibit symptoms of non-bloody diarrhea, abdominal pain, fever, nausea, and muscle pain, known as the disease salmonellosis (Doyle et al. 1997). The FDA reports the infective dose can be as little as 15-20 cells depending on the strain and health of the person infected (FDA 2007). The incubation period is usually between 12 and 72 hours and the illness, usually self limiting, lasts four to seven days (Montville et al. 2005). In rare cases salmonellosis can lead to Reiter's syndrome which consists of pain in joints, irritation of eyes, and painful urination (FDA 2007). Other chronic conditions that can be brought on by *Salmonella* infection include aseptic reactive arthritis and ankylosing spondylitis (Montville et al. 2005). Antibiotics are not typically prescribed, because they prolong carriage within the host, likely due to the repression of natural gut microflora (Montville et al. 2005).

Prevalence and Foodborne Illness

A wide variety of foods have been identified as the source of *Salmonella* in many foodborne outbreaks. This very adaptive pathogen can survive, even grow, in a wide range of foods, and under a wide range of conditions. A short list of the frequently implicated food products includes, but is not limited to, poultry, chocolate, egg salad, raw milk, cantaloupes, ice cream, and fresh produce (Montville et al. 2005; CDC 2007). It is also commonly isolated from swine, cow, and sheep feces, indicating pre-harvest carriage. Undercooked poultry and eggs may be the most important vehicle for *Salmonella* infection (Mastroeni et al. 2006). Poultry consumption has been identified as a risk factor for sporadic cases of *Salmonella* infection (Alterkruse et al. 2006).

It is estimated that *Salmonella* is present on 3-12% of retail chicken sold within the United States (Zhao et al. 2001; FSIS 2007). This is a much lower prevalence than *Campylobacter,* which could be attributed to the regulations that the USDA has in place to help control and test for the presence of *Salmonella* on raw chicken carcasses in slaughter plants. Under the Pathogen Reduction/Hazard Analysis and Critical Control Point final rule, the USDA Food Safety and Inspection Service considers adequate process control to be demonstrated if *Salmonella* contamination of post-chill broiler carcasses is less than 20% (FSIS 2006). FSIS provides an incentive for commercial broiler processors to consistently control *Salmonella* in their slaughter plants, by allowing for a reduction in testing frequency if two sample sets in a row

consistently measure 50% below the performance standard, i.e., six positive samples or less per sample set (FSIS 2006). Currently there is no mandatory testing required for *Campylobacter,* as there is with *Salmonella*. In 2008, the USDA conducted a microbiological sample survey, otherwise known as a baseline study, for the incidence and concentration of *Campylobacter* in broiler chickens. The USDA plans on using the results from the study to help establish a qualitative and quantitative performance standard for *Campylobacter*, possibly during the second half of 2009. Additionally USDA-FSIS has published an updated compliance guidance document for poultry processors to help them control *Salmonella* and *Campylobacter* during production and processing (FSIS 2008b).

Transmission of *Salmonella* and *Campylobacter* during poultry production

Chickens produced on a mass scale for meat consumption are known as broilers. Broilers are raised in grow-out houses with dimensions between 40' and 60' (12-18 m) wide and 300' to 600' (90-180 m) long, housing 12,000 to 35,000 birds per house. Large ventilation fans are used to maintain the optimal temperature. The temperature in the house when the new chicks are first placed starts at around 32˚C (90˚F) (Aviagen 2005). As the chicks age, and are able to maintain their own body temperature, the temperature in the house is decreased until it reaches about the same temperature as the outside environment at the time of depopulation (Aviagen 2005). About once or twice a year the litter is replaced. The litter is made up of wood shavings, peanut hulls, or other

absorbent material. For economic reasons, the litter is generally not changed completely between flocks, but an upper layer may be removed and replaced.

The broilers have continuous access to food and water *ad libitum*. The water is served through a closed system suspended at about the head height of the chickens (Appendix A, Figure 1). Chickens drink by pecking at nipples that dispense drops of water. The feed trays are suspended slightly above the litter and are refilled automatically by machine (Appendix A, Figure 2). The chicks take about six to seven weeks to reach market weight (Axtell 1999).

Avian species have an elevated body temperature which makes them a great reservoir for *Campylobacter* (Skirrow 1977). Both *Salmonella* and *Campylobacter* can be harbored in a chicken's intestinal tract without causing illness, and because of this, during slaughter and further processing they can become a very common source of contamination.

Campylobacter colonization usually does not occur, or cannot be detected, until the chicks are two to four weeks old; this is thought to be because of the presence maternal antibodies in the young chicks (Ketley et al. 2005). Once *Campylobacter* is present in the poultry house, it spreads very rapidly through the entire flock. Many studies have shown that once one bird has been colonized the rest of the flock follows very quickly; usually close to 100% of the birds are colonized by the time they are sent to slaughter (Keener et al. 2004). Van Gerwe et al. (2008) estimated the rate of infection was 2.37 infections per infectious bird per day. At that rate, 95% of the flock would be colonized in seven days or less.

There are a variety of routes through which a flock can become colonized with *Campylobacter* and/or *Salmonella*. Environmental sources play a significant role in spreading the pathogens throughout the flock (Jacobs-Reitsma et al. 1995; Hiett et al. 2002). Many studies have examined a variety of modes of transmission within a flock. Berrang et al. (2003) recovered *Campylobacter* spp. from the respiratory tracts of broilers before scalding, while Lues et al. (2007) found *Salmonella* spp. in the air of several chicken slaughter facilities. Hald et al. (2007) theorizes that flies play a major role in the introduction of *Campylobacter* into a flock; he found a 40% decrease in positive flocks when fly screens were implemented.

Airborne bacteria may play a role in transmission. Harbaugh et al. (2006) showed that turkeys in holding sheds could be infected with *Salmonella enterica* serovar Typhimurium after two hours of exposure to circulating air containing high levels of bacteria from infected feces. A different study also showed that it was possible for *Salmonella* enterica serotypes Enteriditis and Typhimurium to survive in aerosols for at least two hours (McDermid et al. 1996). In a later related study, Lever went on to show that *Salmonella* could be transmitted in the air between chicks that were in separate rooms connected only by an air vent (Lever et al. 1996). Another study showed that birds infected with aerosolized *Salmonella* increased contamination of eggs and muscle tissue, despite being administered less CFUs per bird than compared to orally infected birds (Leach et al. 1999). Venter et al. (2004) sampled inside automated egg management facilities and concluded that the main source of bioaerosols was from the

chickens themselves. Interestingly enough, Bailey and Cosby (2005) concluded that free-range and organic chickens were no less likely to carry or be infected with *Salmonella* than conventionally raised chickens.

Chickens are known to dig into the litter, peck at the ground, and at times, eat feces. A potential means of spread for *Campylobacter* is the spent litter that is not cleared out between flocks (Line et al. 2006). Low surface humidity of litter can lead to lower surface contamination of litter with *Salmonella*, because of the dryness, and thus lead to lower contamination on carcasses (Mallinson et al. 1998). As stated earlier, other animals (duck, sheep, pork, dairy and beef cattle) besides chickens harbor *Campylobacter*, and as such would shed the pathogen in their feces (Devane et al. 2004). These and other potential environmental sources could be spread into the poultry house by farm workers, their equipment, or their transports vehicles (Johnsen et al. 2006).

Unlike *Campylobacter*, *Salmonella* can be transferred vertically from hen to egg, i.e., the *Salmonella* bacteria can become internalized in eggs. This occurs if a hen's ovaries are infected, the bacteria can then contaminate the egg before the shell has been formed (Braden 2006). Although there are external disinfection methods in place to clean the egg's shells they do nothing to the viability of the internalized bacteria (Hope et al. 2002).

Current Testing Procedures for *Campylobacter* and *Salmonella*

Most studies of *Campylobacter* use different selective plating media and subsequent biochemical tests to confirm its species. According to the FDA's

Bacteriological Analytical Manual (BAM), most food samples that are to be analyzed for *Campylobacter* need to go through a pre-enrichment, enrichment, plating onto isolation agar, and multiple confirmation tests before confirming a positive sample (FDA 2007b). One example is an antibody based test such as the latex agglutination test which gives a presumptive identification of *Campylobacter* spp. The FDA BAM method utilizes modified charcoal cefoperazone deoxycholate agar (mCCDA), which uses charcoal instead of blood (Stern et al. 2001). This is a common media used in many studies because it not only selects for *Campylobacter* spp., it is also easy to see the white colonies on the black colored media. Other commonly used plating media are Campy-Cefex, and Campy-Line agar (Potturi-Venkata et al. 2007). Phenotypic subtyping of *Campylobacter* is not commercially available but antigens can be requested for approved uses from the *Campylobacter* Reference Laboratory, Centers for Disease Control (Stern et al. 2001).

Microscopic examination of cells is a simple and quick way to determine the existence of *Campylobacter* cells in a sample or from growth on a plate. The cells are distinguished by their spiral rod or seagull shape. Although this form can become altered, forming into a spherical or coccoidal shape, by exposure to oxygen for an extended period of time (Montville et al. 2005).

For *Salmonella*, conventional testing procedures can take five days for presumptive results (FDA 2007). When testing for *Salmonella*, FDA's BAM manual calls for the use of lactose broth before transfer to selenite cystine broth or Rappaport-Vassiliadis broth (FDA 2007). After incubation of the broth the

samples are streaked for isolation onto three different selective agars, bismuth sulfite agar, xylose lysine desoxycholate agar, and Hektoen enteric agar. There are many biochemical tests that are to be performed to confirm a positive sample for *Salmonella*. One of these is the Pullorum-Gallinarum serological test, for which certain vaccines against *S.* Enteritidis can cause false positive reactions (OIE 2008).

Culture-based methods have a number of pitfalls and limitations, they are relatively slow, they can be confounded by high background growth, and enrichment broth is usually needed to recover small numbers of cells from the tested sample (Moore 2005, Steffan 1991). All of this can require an entire work week to reach a positive result. Detection, identification, and quantification of foodborne pathogens can become difficult if there are low numbers of pathogens present or if there is interference from a food matrix (Maurer 2006). An organism's genetic makeup determines how it causes disease; many of these virulence genes are specific to one particular pathogen, and because of this they can be used to identify that pathogen using a molecular based technique known as the polymerase chain reaction or PCR (Maurer 2006). DNA-based methodologies (e.g., traditional PCR and real-time PCR) are rapidly becoming the method of choice for pathogen detection and quantification in laboratory settings because it is becoming easier, more mainstreamed and streamlined, and routinely used by government agencies such as the FDA and CDC in outbreak investigations (CDC 2007). Use of PCR assays provides a specific, sensitive, and rapid method for detection of all types of bacterial pathogens.

There are many genes that can be used as target genes for PCR analysis. Some genes all bacteria must possess to grow or survive, they are referred to as housekeeping genes such as 16s rRNA (Case et al. 2007). For a PCR assay to be effective the target gene sequence must be specific to the organism that is to be detected, such as a unique part of the 16s rRNA. Virulence genes can also be very helpful when designing primers to detect pathogenic bacteria. The cytolethal distending toxin (cdtB) gene in *Campylobacter* or the invA gene in *Salmonella* are examples highly specific virulence genes that are often targeted (Maurer 2006; Asakura et al. 2008). This targeted sequence will not only be unique to the bacterium but it will also have a specific molecular weight that will be utilized when visualizing the DNA during qualitative analysis.

Once the bacteria have been collected in a sample, the DNA has to be extracted and purified, which can be done a variety of ways. Use of enrichment, immounocapture, or buoyant density centrifugation concentrates the bacterial sample, while the use of heat, detergents, or chemical solvents can be used to extract the DNA from bacterial cells (Maurer 2006). Once the DNA, known as the template, has been extracted, it is ready for amplification. This multiplies the amount DNA so that it reaches a level that can be visualized. Only a specific fragment of the DNA is amplified using unique primers that anneal to specific priming regions of the DNA (Saiki et al. 1988).

After the DNA is amplified, now known as the amplicon, it is visualized by gel electrophoresis. The targeted sequence of DNA has a unique molecular weight that distinguishes it from other pieces of DNA. This uniqueness is used to

determine positive or negative results when looking at the amplicon in the gel. A sample is only considered positive if the amplicon has the expected fragment size for the primers used (Maurer 2006).

Many studies claim that PCR analysis is sensitive enough to detect a single bacterial cell per in a given environmental sample. While this may be true, it can be difficult to actually extract that single cell from the original sample, so enrichment cultures are utilized to amplify the numbers of viable cells before use of the PCR assay (Moore 2005). Another important point is that conventional PCR cannot differentiate between living and dead cells (Josephson et al. 1993). There have been studies looking at different methods to distinguish between viable and non-viable cells, such as use of messenger RNA, or ethidium monoazide, but they have had very variable results (Bej et al. 1996; Rudi et al. 2005) and additional studies need to be conducted before these techniques could reliably be incorporated into investigations. PCR methods for the detection of *Campylobacter*, *Salmonella,* and other pathogens during food processing could be used to determine points in the food production process where contamination occurs and where controls could be introduced to reduce or eliminate it from retail food products, thereby reducing the risk to the consumer (Moore 2005).

Current pre-slaughter testing and regulations

The U.S. federal government does not have *Campylobacter* or *Salmonella* testing regulations for commercial poultry flocks. However, it does have

guidelines dealing with biosecurity (FSIS 2006). Poultry producers are more concerned with avian influenza (AI) as it has the potential to wipe out an entire flock, with death occurring within two to three days after infection (Doyle et al. 2006). They may also be less concerned with foodborne pathogens since there are post-harvest control measures used in the slaughter or further processing plants that can reduce the prevalence or concentration of human pathogens. Many guidelines for biosecurity put out by various governments not only apply to control of AI but also to the control of human pathogens that are found in the animals being reared. For instance, the Codex Alimentarius Commission recommends that any equipment to be taken into poultry houses should be sanitized beforehand (WHO 2008). Implementing Good Hygienic Practices (GHP), biosecurity measures, and Hazard Analysis and Critical Control Points (HACCP) are additional ways to control *Campylobacter* on the farm (Moore 2005). The Terrestrial Animal Health Standards Commission recommends sampling flocks at least once before slaughter for the testing of *Salmonella* (OIE 2008).

Environmental sampling procedures for *Campylobacter* and *Salmonella*

Many studies use dissection and testing of the chicken's intestinal tract as a means of determining whether a bird has become colonized by a pathogen of interest. When processing carcasses it can be very beneficial to know where the majority of these pathogens are, so that safety measures can be enacted to prevent and reduce contamination. To accomplish this before the birds reach the

slaughter plant, many studies use invasive animal testing, such as dissection of birds, to test the intestinal tracts, crops, or other such organs within the birds (Cox et al. 2007; Rasschaert et al. 2007). Environmental sampling has been used as a means to identify potential reservoirs and routes of transmission of these pathogens within the poultry environment. Rambau et al. (2004) sampled formites, inanimate objects which bacteria are capable of surviving on, and found that catcher's boots, forklift wheels, and crates were just some of a multitude of objects that could introduce or spread *C. jejuni* within the poultry house.

Many environmental studies have used drag swabs, or some variation thereof, to sample for the presences of pathogens. Hansson et al (2007) found *Campylobacter* using cloth bandages wrapped around investigator's shoes worn inside poultry houses. Liu et al. (2002) and Soumet et al. (1999) used drag swabs in combination with a PCR assay to detect *Salmonella* within poultry houses. *Campylobacter* and *Salmonella* have been found practically anywhere in or around the poultry house or farm environment.

Air Sampling

There have been few studies looking at the role of airborne transmission of pathogens, especially with regard to pathogens that are highly sensitive to oxygen, such as *Campylobacter*. Many studies have been able to recover *Campylobacter* in poultry houses and in processing plants when using a passive settling method, allowing airborne particulates to naturally settle on to agar plates for a set period of time (Berndtson et al. 1996; Bull et al. 2006). Alvarez et al. (1995) suggested that air sampling coupled with PCR analysis could be used to

identify airborne *E.coli* and *Shigell.* But samples with 10^3 to 10^4 CFU environmental organisms caused inhibition during amplification but this could be remedied by a 1:10 dilution (Alvarez et al. 1995). Olsen et al (2009) was able to use an electrostatic capture air sampling method where only 1800 ml needed to be collected to detect *Campylobacter* with real time PCR.

Sartorius Stedim Biotech, a company based in Germany, produces a special gelatin filter for air sampling, which has been used mainly in medical and pharmaceutical environments. The filter can be easily dissolved in a buffer or enrichment broth, or it can be placed directly onto an agar plate for direct enumeration. Studies involving these filters have been used to monitor the airborne movement of anthrax spores in an outdoor environment (P.Turnbull 1998), or used in the detection of airborne *Chlamydophila psittaci* (Van Droogenbroeck et al. 2009). Schwerwing et al. (2007) found that the filters could be used on a pharmaceutical aseptic fill line to collect sample continuously for 8 hours without causing a significant difference in overall microbial diversity or total recovery as compared to controls (Scherwing et al. 2007). Although these filters were originally intended for use in medical clean rooms they might be used in a food safety setting to collect environmental samples of *C. jejuni* and *Salmonella*.

REFERENCES

Allos, B. (2001). "*Campylobacter jejuni* infections: Update on emerging issues and trends." Clin. Infect. Dis. **32**(8): 1201-1206.

Altekruse, S., N. Stern, P. Fields and D. Swerdlow (1999). "*Campylobacter jejuni* - an emerging foodborne pathogen." Emerg. Infect. Dis. **5**(1): 28-35.

Alterkruse, S., N. Bauer, A. Chanlongbutra, R. DeSagun, A. Naugle, W. Schlosser, R. Umholtz and P. White (2006). "*Salmonella* Enteritidis in broiler chickens, United States, 2000-2005." Emerg. Infect. Dis.**12**(12): 1848-1852.

Asakura, M., W. Samosornsuk, A. Hinenoya, N. Misawa, K. Nishimura, A. Matsuhisa and S. Yamasaki (2008). "Development of a cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the detection and identification of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus*." FEMS Immunol. Med. Mic. **52**(2): 260-266.

Axtell, R. (1999). "Poultry integrated pest management: Status and future." Integrated Pest Management Reviews **4**(1): 53-73.

Aviagen. (2005). Environmental Management in the Broiler-Breeder Rearing House. Retrieved April 24, 2009, from http://www.aaf.com/docs/RearingHse_FINAL.pdf

Bej, A., N. Wee-Yao, S. Morgan, D. Jones and M. Mahbubani (1996). "Detection of viable *Vibrio cholerae* by reverse-transcriptase polymerase chain reaction [rt-pcr]." Molecular Biotechnology **5**(1): 1-10.

Berndtson, E., M. Danielsson-Tham and A. Engvall (1996). "*Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process." Int. J. Food Microbiol. **32**: 35-47.

Braden, C. (2006). "*Salmonella* enterica serotype enteritidis and eggs: A national epidemic in the United States." Clin. Infect. Dis. **43**(4): 512-517.

Brenner, F., R. Villar, F. Angulo, R. Tauxe and B. Swaminathan (2000). "*Salmonella* nomenclature." J. Clin. Microbiol. **38**(7): 2465-2467.

Bull, S., V. Allen, G. Domingue, F. Jorgensen, J. Frost, R. Ure, R. Whyte, D. Tinker, J. E. Corry, J. Gillard-King and T. Humphrey (2006). "Sources of *Campylobacter* spp. Colonizing housed broiler flocks during rearing." Appl. Environ. Microbiol. **72**(1): 645-652.

Case, R., Y. Boucher, I. Dahllof, C. Holmstrom, W. Doolittle and S. Kjelleberg (2007). "Use of 16s rRNA and rpoB genes as molecular markers for microbial ecology studies." Appl. Environ. Microbiol. **73**(1): 278-288.

CDC (2007) "Multistate outbreaks of *Salmonella* infections associated with raw tomatoes eaten in restaurants—United States 2005-2006." MMWR 56(35): 909-911.

CDC. (2008). "Foodnet facts and figures - number and incidence of top 10 *Salmonella* serotypes in 2007." Retrieved Feb 14, 2009, from http://www.cdc.gov/FoodNet/factsandfigures/6.pdf.

Cox, N., L. Richardson, R. Buhr, J.Northcutt, J. Bailey, P. Cray and K. Hiett (2007). "Recovery of *Campylobacter* and *Salmonella* serovars from the spleen, liver and gallbladder, and ceca of six- and eight-week-old commercial broilers." J. Appl.Poult. Res. **16**(4): 477-480.

Devane, M. L., C. Nicol, A. Ball, J. D. Klena, P. Scholes, J. A. Hudson, M. G. Baker, B. J. Gilpin, N. Garrett and M. G. Savill (2005). "The occurrence of *Campylobacter* subtypes in environmental reservoirs and potential transmission routes." J Appl Microbiol **98**(4): 980-90.

Doyle, M., L. Beuchat and T. Montville (1997). *Salmonella* species. Food microbiology: Fundamentals and frontiers. ASM Press, Washington D.C., 129-158.

Doyle, M. and M. Erickson (2006). "Emerging microbiological food safety issues related to meat." Meat Science **74**(1): 98-112.

ERS. (2008). "U.S. Broiler industry: Background statistics and information." Retrieved April 10, 2009, from http://www.ers.usda.gov/News/broilerCoverage.htm.

FDA. (2007a). "*Campylobacter jejuni*." Bad Bug Book Retrieved April 22, 2008, from http://www.cfsan.fda.gov/~mow/chap4.html.

FDA. (2007b). "*Salmonella*." Bacteriological Analylitic Manual Online Retrieved April 10, 2008, from http://www.cfsan.fda.gov/~ebam/bam-5.html.

FDA. (2007c). "*Salmonella* spp." Bad Bug Book Retrieved April 22, 2008, from http://www.cfsan.fda.gov/~mow/chap1.html.

Friedman, C., J. Neimann, H. Wegener and R. Tauxe (2000). "Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations" In: Campylobacter, 2nd edition, Nachamkin, I, Blaser, MJ (Eds), Am Soc Microbiol, Washington DC.

FSIS. (2008b). "Compliance guideline for controlling *Salmonella* in poultry." Retrieved Feb 13, 2009, from

http://www.fsis.usda.gov/PDF/Compliance_Guideline_Controlling_*Salmonell a*_Poultry.pdf.

FSIS (2008a). "Young Chicken Baseline Data Collection Program." Retrieved April 20, 2009, from http://www.fsis.usda.gov/PDF/Young_Chicken_Baseline_Data_Collection.pdf .

FSIS. (2006). *Salmonella* verification sample result reporting agency policy and use in public health protection, Federal Register. **71:** 9772-9227.

FSIS. (2007). "Quarterly progress report on *Salmonella* testing of selected raw meat and poultry products: Preliminary results, July - September 2008 " Retrieved Feb 13, 2009, from http://www.fsis.usda.gov/PDF/Q3_2008_*Salmonella*_Testing.pdf.

Hansson, I., I. Vagsholm, L. Svensson and E. Olsson Engvall (2007). "Correlations between *Campylobacter* spp. Prevalence in the environment and broiler flocks." J. Appl. Microbiol. **103**(3): 640-649.

Hazeleger, W., J. Wouters, F. Rombouts and T. Abee (1998). "Physiological activity of *Campylobacter* jejuni far below the minimal growth temperature." Appl. Environ. Microbiol. **64**(10): 3917-3922.

Hiett, K., N. Stern, P. Fedorka-Cray, N. Cox, M. Musgrove and S. Ladely (2002). "Molecular subtype analyses of *Campylobacter* spp. From Arkansas and California poultry operations." Appl. Environ. Microbiol. **68**(12): 6220-6236.

Holler, C., D. Witthuhn and B. Janzen-Blunck (1998). "Effect of low temperatures on growth, structure, and metabolism of *Campylobacter* coli SP10." Appl. Environ. Microbiol. **64**(2): 581-587.

Hope, B., A. Baker, E. Edel, A. Hogue, W. Schlosser, R. Whiting, R. McDowell and R. Morales (2002). "An overview of the *Salmonella* enteritidis risk assessment for shell eggs and egg products." Risk Anal. **22**(2): 203-218.

Jacobs-Reitsma, W., A. Giessen, N. Bolder and R. Mulder (1995). "Epidemiology of *Campylobacter* spp. At two dutch broiler farms." Epid.Infection **114**(3): 413-421.

Johnsen, G., H. Kruse and M. Hofshagen (2006). "Genetic diversity and description of transmission routes for *Campylobacter* on broiler farms by amplified-fragment length polymorphism." J. Appl. Microbiol. 101(5):1130-1139.

Josephson, K., C. Gerba and I. Pepper (1993). "Polymerase chain reaction detection of nonviable bacterial pathogens." Appl. Environ. Microbiol. **59**(10): 3513-3515.

Keener, K., M. Bashor, P. Curtis, B. Sheldon and S. Katharlou (2004). "Comprehensive review of *Campylobacter* and poultry processing." CRFSFS **3**(2): 105-116.

Ketley, J. and M. Konkel (2005). *Campylobacter*: Molecular and Cellular Biology. Horizon Bioscience, Wymondham, UK..

Leach, S., A. Williams, A. Davies, J. Wilson, P. Marsh and T. Humphrey (1999). "Aerosol route enhances the contamination of intact eggs and muscle of experimentally infected laying hens by *Salmonella* Typhimurium dt104." FEMS Microbiol. Lett. **171**(2): 203-207.

Leonard II, E., L. Tompkins, S. Falkow and I. Nachamkin (2004). "Comparison of *Campylobacter* jejuni isolates implicated in guillain-barre syndrome and strains that cause enteritis by a DNA microarray." Infect. Immun. **72**(2): 1199-1203.

Lever, M. and A. Williams (1996). "Cross-infection of chicks by airborne transmission of *Salmonella* enteritidis pt4" Lett. Appl. Microbiol. **23**(5): 347-349.

Line, J. (2006). "Influence of relative humidity on transmission of *Campylobacter* jejuni in broiler chickens." Poult. Sci. **85**(7): 1145-1150.

Line, J. E. and J. S. Bailey (2006). "Effect of on-farm litter acidification treatments on *Campylobacter* and *Salmonella* populations in commercial broiler houses in northeast Georgia." Poult. Sci. **85**(9): 1529-1534.

Mastroeni, P. and D. Maskell (2006). *Salmonella* infections: Clinical, immunological, and molecular aspects. In: Advances in Molecular and Cellular Microbiology. Cambridge University Press, Cambridge, UK.

Maurer, J. (2006). PCR Methods in Foods. New York, New York, Springer.

McDermid, A. and M. Lever (1996). "Survival of *Salmonella* Enteritidis pt4 and *Salmonella* Typhimurium Swindon in aerosols." Lett. Appl. Microbiol. **23**(2): 107-109.

Montville, T. and K. Matthews (2005). Food Microbiology: An Introduction. Washington D.C., ASM Press.

Moore, J. (2005). "*Campylobacter*." Vet. Res. **36**(3): 351-382.

OIE (2008). Guidelines on the detection, control, and prevention of *Salmonella* spp. in poultry. Terrestrial Animal Health Standards Commission. http://www.aphis.usda.gov/import_export/animals/oie/downloads/tahc_mar-sep08/tahc-guide-det-con-prev-spp-poultry-77-mar08.pdf. Retrieved February 10, 2009.

Olsen, K., M. Lund, J. Skov, L. Christensen and J. Hoorfar (2009). "Towards real-time monitoring of broiler flocks: Detection of *Campylobacter* in air samples for continuous monitoring of *Campylobacter* colonization in broiler flocks." Appl. Environ. Microbiol.: AEM.02182-08.

Potturi-Venkata, L., S. Backert, A. Lastovica, S. Vieira, R. Norton, R. Miller, S. Pierce and O. Oyarzabal (2007). "Evaluation of different plate media for direct cultivation of *Campylobacter* species from live broilers." Poult. Sci. **86**(7): 1304-1311.

Ramabu, S., N. Boxall, P. Madie and S. Fenwick (2004). "Some potential sources for transmission of *Campylobacter* jejuni to broiler chickens." Lett. Appl. Microbiol. **39**(3): 252-256.

Rasschaert, G., K. Houf, J. Van Hende and L. De Zutter (2007). "Investigation of the concurrent colonization with *Campylobacter* and *Salmonella* in poultry flocks and assessment of the sampling site for status determination at slaughter." Vet. Microbiol. **123**(1-3): 104-9.

Rudi, K., B. Moen, S. Dromtorp and A. Holck (2005). "Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples." Appl. Environ. Microbiol. **71**(2): 1018-1024.

Saiki, R., D. Gelfand, S. Stoffel, S. Scharf, R. Higuchi, G. Horn, K. Mullis and H. Erlich (1988). "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase." Science **239**(4839): 487-491.

Scherwing, C., F. Golin, O. Guenec, K. Pflanz, G. Dalmaso, M. Bini and F. Andone (2007). "Continuous microbiological air monitoring for aseptic filling lines." PDA J Pharm Sci Technol. **61**(2):102-9.

Skirrow, M. (1977). "*Campylobacter* enteritis: A "New" Disease." Brit. Med. J.: **2**(6078): 9–11.

Snelling, W., M. Matsuda, J. Moore and J. Dooley (2005). "*Campylobacter jejuni*." Lett.Appl.Microbiol. **41**(4): 297-302.

Soumet, G., V. Rose, N. Rose, P. Drouin, G. Salvat, P. Colin. (1999). "Identification by a multiplex PCR-based assay of *Salmonella* Typhimurium

and *Salmonella* Enteritidis strains from environmental swabs of poultry houses." Lett. Appl. Microbiol. **29**(1): 1-6.

Steffan, R. and R. Atlas (1991). "Polymerase chain reaction: Applications in environmental microbiology." Ann. Rev. Microbiol. **45**(1): 137-161.

Stern, N., J. Line and H. Chen (2001). *Campylobacter*. In: Compendium of Methods for the Microbiological Examination of Foods. F. Downes and K. Ito. Washington D.C., American Public Health Association**:** 301-310.

Stern, N. J. and M. C. Robach (2003). "Enumeration of campylobacter spp. In broiler feces and in corresponding processed carcasses." Journal of Food Protection **66**: 1557-1563.

Turnbull, P., J. Le Roux, A. Bennett, S. Parks (1998). "Airborne movement of anthrax spores from carcass sites in the Etosha National Park, Namibia." J. Appl. Microbiol. **84**(4): 667-676.

Van Droogenbroeck, C., M. Van Risseghem, L. Braeckman and D. Vanrompay (2009). "Evaluation of bioaerosol sampling techniques for the detection of *Chlamydophila psittaci* in contaminated air." Vet. Microbiol. **135**(1-2): 31-37.

WHO (2008). Proposed Draft Guidelines for Control of *Campylobacter* and *Salmonella* spp. In Chicken Meat at Step 3: Report of the 40th session of the codex committee on food hygiene. Guatemala City, Guatemala Accessed from: ftp://ftp.fao.org/codex/ccfh40/fh40_06e.pdf

Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. White, D. Wagner and J. Meng (2001). "Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the greater Washington, D.C. area." Appl. Environ. Microbiol. **67**(12): 5431-5436.

CHAPTER TWO

Campylobacter jejuni and *Salmonella* spp. Detection in Chicken Grow Out Houses by Environmental Sampling Methods

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ABSTRACT

Campylobacter and *Salmonella* are foodborne pathogens commonly associated with raw poultry. Although there has been much research done on isolating these pathogens from poultry production environments using cloacal swabs, fecal samples, intestinal tract contents and dissection, research involving environmental sampling has been limited. New and/or improved environmental sampling methods may provide an easy, convenient, and less time-consuming way to collect samples. Coupling these sampling methods with PCR may provide a relatively simple, rapid, and robust means of testing for foodborne pathogens in a chicken house or flock prior to slaughter.

Air, boot and sponge samples were collected from three commercial chicken grow-out houses located in southwestern Virginia when flocks were three, four, and five weeks old. Air samples were collected onto gelatin filters. Fecal/litter samples were collected from disposable booties worn over investigator's protective shoe coverings. Pre-moistened sponges were used to sample house feed pans and water dispensers on drink lines. A PCR method was used to qualitatively detect *Campylobacter jejuni* and *Salmonella* spp. *Campylobacter jejuni* was detected at each farm (house), across all three ages (3, 4, and 5 weeks), and from each sample type. *Salmonella* was not detected in any of the environmental samples. For all 270 samples, 41% (110/270) were positive for *Campylobacter.* Collectively, 28% (25/90) of air, 44% (40/90) of sponge, and 50% (45/90) of bootie samples were positive for *Campylobacter*. The methods used in this study are non-invasive to live animals, relatively rapid

and specific, and could enable poultry processing facilities to coordinate scheduled processing of flocks with lower pathogen incidence, as a way to reduce post-slaughter pathogen transmission.

INTRODUCTION

The United States poultry industry produced 36.1 billion pounds of chicken in 2007, with Americans consuming 86 pounds per person in 2006 (ERS 2008). To handle this huge demand chickens are raised in large flocks, usually numbering more than 30,000 chicks at start, in large grow out houses. This large scale rearing makes it very easy for pathogenic bacteria to spread within a flock. While *Campylobacter* and *Salmonella* do not cause illness in birds they do have a serious impact on human health and are the most important foodborne pathogens associated with raw or undercooked poultry meat. The US Department of Agriculture (USDA) currently has regulations governing proper control of *Salmonella* within slaughter plants but there are no regulations or testing done before slaughter, at the farm level. Although *Campylobacter* is estimated to be the leading cause of bacterial gastroenteritis (Moore 2005) the USDA has no mandates for its control in poultry processing and production. The USDA Food Safety and Inspection Service (FSIS) have been conducting baseline studies since 2006 to investigate the prevalence and quantitative level of *Campylobacter* on broiler carcasses within slaughter facilities (FSIS 2008a). The data collected from these studies will be used to develop standards for process control of *Campylobacter* which may become mandatory by the end of 2009. The USDA does try to help farmers reduce the prevalence of *Campylobacter* and *Salmonella* within their flocks by providing voluntary guidelines for controlling these pathogens (FSIS 2008b).

Poultry carcasses and processed poultry products can become cross-contaminated or newly contaminated with harmful bacteria at many points during the process of growing, slaughtering, and processing of raw poultry. Since a typical commercial chicken plant will process 10,000 to 20,000 birds per hour for 16 hours a day, cross-contamination is virtually impossible to eliminate. Many poultry processors employ measures to reduce or eliminate pathogens on the poultry carcasses during processing. Antimicrobial sprays or dips are sometimes used to reduce the pathogenic load on the exterior of the poultry carcasses before they are dropped into a chilled water tank. The chill tank is a step that helps to reduce pathogens since this cold water bath usually contains chlorine. The USDA FSIS requires industry to reduce chicken carcass temperatures to 4˚C within four hours since rapid cooling will prevent the growth of *Salmonella* and other microorganisms. Many processors have increased their usage of chlorine and rinse water volumes to reduce *Salmonella* and *Escherichia coli* on carcasses (Arritt et al. 2002). Some companies attempt to control *Salmonella* at the earliest stage possible by vaccinating newly hatched broiler chicks. These strategies may have some effect for reducing *Campylobacter* populations, but limited research has been done to demonstrate other process interventions that are effective for reducing both the prevalence and concentration of this pathogen. Even with these pathogen reducing interventions *Campylobacter* has still been found on 30-100% of retail chicken carcasses while *Salmonella* has been found on up to 40% *(*Altekruse et al. 1999, Zhao et al. 2001). Interventions on the farm level, when

the chickens are being raised, may be a way to further reduce the amount of harmful bacteria found on raw chicken products on the market.

The routes of transmission for which *Campylobacter* and *Salmonella* are spread throughout a flock are not fully understood. Consequently, control and eradication of these pathogens is difficult once they are present within a poultry house. Chickens are able to harbor these pathogens asymptomatically; therefore internal organs of chickens are often tested to confirm a positive infection within a flock (Cox et al. 2007). The chickens also excrete the pathogens in their stool so it could generally be assumed a flock is infected if *Campylobacter* or *Salmonella* are found within the poultry house in fecal droppings (Stern et al 2003). Sampling in and around the poultry environment has been conducted to try to find reservoirs where these pathogens survive and to try to find answers as to how they are transmitted throughout entire flocks in relatively short time periods (Berndtson 1996, Devane 2005). Controlling the spread of these pathogens within flocks could help to make our food supply safer by reducing the amount of harmful bacteria going through the slaughter plants.

In a previous unpublished study, air from chicken grow out houses was collected directly onto selective agar plates (mCCDA and Campy-Cefex). Campylobacter was not detected from any air samples from within the poultry houses but some was detected in the slaughter plant. Although very few plates were able to grow *Campylobacter,* it is still highly likely that the pathogen is present in the poultry house environment but may not have been healthy enough to readily grow on the selective plates. By utilizing a different type of air

collection media (gelatin filters) and two other methods of sample collection (sponge and fecal), detection of *Campylobacter* and/or *Salmonella* should be greatly increased. We hypothesize that if *Campylobacter* and/or *Salmonella* are detected in swab samples or fecal samples then it will also be found in the air. We would also expect to see an increase of positive air samples as the age of the birds increase.

The purpose of this study is to use environmental sampling to detect the presence of *Campylobacter* and *Salmonella* spp. in chicken grow out houses. By incorporating the use of air sampling, swab sampling, and fecal samples, the condition of the flock can be monitored for colonization of either pathogen, *Campylobacter* or *Salmonella*, quickly and easily without the need to physically inspect any of the chickens. This could, in turn, allow processors to reduce their overall incidence of the pathogens within the slaughter plant.

MATERIALS AND METHODS

Sample Sites

Three commercial chicken farms in Virginia were selected as sample sites based on when new flocks were placed. Sample collection was performed between the months of October and December in 2008. One poultry grow-out house per farm was randomly selected to sample. Each house was approximately 400' (122 m) long and 50' (15 m) wide and contained approximately 37,000 birds per flock. These same three poultry houses were sampled when the birds were three weeks, four weeks, and five weeks old.

Sample Collection

The poultry house was spatially divided into 10 equal sections to designate where sampling was to occur. As directed by their biosecurity policies, poultry farm operators provided the investigators with clean coveralls, hairnets, and plastic boot covers. All sample collection, preparation, and transfers were done within the poultry house. All samples were returned to the laboratory for analysis within 24 hours of collection.

Air Sampling

Ten air samples (1000 L each collected at 50 L/min) were collected in each poultry house. Air was collected onto gelatin membrane disposable filters (Microbiology International, Frederick, MD) using the Sartorius Airport MD8 air sampler (Microbiology International, Frederick, MD). Samplers were placed along

walls two to four feet above the ground. Filters were removed from the air sampler using sterile disposable tweezers and placed in 99 ml of Butterfield's phosphate dilution buffer (BPDB) (prefilled dilution bottles, Remel, Lenexa, KS) and put on ice until returned to lab for analysis.

Swab sampling

Ten swab samples were collected in each poultry house. Three feeder pans and four watering nipples were swabbed per sponge. A sterile 2"x1" (5cm x 2.5 cm) sponge was pre-wetted with 10 ml phosphate buffer solution. Feed pans were swabbed with wetted sponge on all areas except those that contacted the ground, totaling about 100 cm^2. The used sponges were then sealed in sterile whirl-pack bags and placed on ice until returned to lab for analysis.

Fecal/litter Sampling

Ten fecal/litter samples were collected in each poultry house from each of the 10 sections. Clean booties (DuPont Gripper clean room shoe covers; Fisher Scientific) were placed over outer protective footwear and worn for 2 min as investigators walked in a designated section. Booties were then removed, placed in 400 ml phosphate buffer, sealed in a large whirl-pack bag, and massaged for 1 minute to evenly wash bootie in buffer solution. They were then placed on ice until returned to lab for further analysis.

Microbial Analysis

Enrichment

For *Campylobacter*, BPDB solutions (1 ml) from each sample were pipetted into 10 ml of Bolton Selective Enrichment Broth (Oxoid Hampshire, England) with Bolton Broth Selective Supplement (10 mg cefoperazone, 10mg vancomycin, 10 mg trimethoprim, and 25 mg cycloheximide per vial) and 5% laked horse blood (Remel, Lenexa, KS). Tubes of broth were then incubated under micro-aerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C for 48±2 hrs. Micro-aerophilic conditions were obtained by placing one Pack-MicroAero gas pack (Mitsubishi Gas Chemical Company Inc. New York, NY) into an anaerobe jar with the broth tubes.

For *Salmonella*, 1 ml of liquid sample was pipetted into 10 ml of 1x lactose broth (BD, Sparks, MD). Tubes of broth were incubated in normal atmospheric conditions at 35ºC for 48±2 hrs.

Control Cultures

For PCR analysis, of *C. jejuni* ATCC 29428 was obtained from MicroBiologics in St. Cloud, MN. Culture was revived in Bolton's broth and plated onto Brucella agar (BD, Sparks, MD) for visual inspection of purity. Culture was then re-inoculated into fresh Bolton's broth and treated in the same manner as the samples described above. *Salmonella* Typhimurium x3181 culture was obtained from the ultra-low freezer at the Department of Food Science and Technology at Virginia Tech. Culture was revived in tryptic soy

broth (Difco, Franklin Lakes, NJ), incubated at 35˚C for 48 hours then treated in the same manner as the samples described above.

DNA Extraction and Purification

DNA was extracted using the Qiagen Generation Capture Plate Kit per manufacturers' instructions for cell suspensions (Qiagen, Valencia, CA). *Campylobacter* and *Salmonella* samples were processed in the same manner. Briefly, 1 ml of enriched broth was pipetted into one well on a 96 well plate. Once all wells were filled the plate was centrifuged for 10 minutes at 1,000 rpm. The supernatant was removed and the pellet was re-suspended in 200 µl phosphate buffer solution. DNA was extracted following manufacturer's protocols, but the plates were microwaved for 14 minutes at 20% power instead of 28 minutes. Upon completion the sample collection plate was placed at -20ºC until further analysis.

DNA Amplification

PCR was performed to amplify *Campylobacter* and *Salmonella* specific sequences present in the DNA samples. A 23 µl mastermix for each sample was prepared. It included the following: 2.5 µl 10x PCR Buffer (100 mM Tris-HCl pH 9.0) (Fisher Bioreagents, Springfield, NJ), 0.5 µl dNTPs (10 mM each of dATP, dCTP, dGTP, dTTP) (Qiagen, Valencia, CA), 2.5 µl of each primer (5uM) (the primers used are described in the section following.), 0.2 µl (1U) Taq polymerase (Fisher Bioreagents, Springfield, NJ), 2.5 µl 10% DMSO (Fisher, Springfield, NJ) 12.3 µl dH_2O (Fisher, Springfield, NJ)and 2 µl of purified DNA template was added to give a total reaction volume of 25 µl. The PCR analysis was performed

in an iCycler thermal cycler (Bio-Rad Hercules, CA) using the following protocol: initial denaturation at 95˚C for 5 minutes, followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 56˚C for 30 seconds, and extension at 72˚C for 1 minute, then finished with a final extension at 72˚C for 10 minutes and held at 4˚C until next step. Ten µl of each PCR product was loaded into a 1.5% agarose gel containing ethidium bromide and analyzed after gel electrophoresis (45 mins at 80 volts); bands were visualized with UV light. Positive samples were marked for *C. jejuni* if a band was seen at 510 bp in relation to the reference ladder, whereas positive samples for *Salmonella* were marked if a band showed at 1935 bp.

DNA Primers

All primers were obtained from Integrated DNA Technologies located in Coralville, IA. The primers were designed using muPlex Multi-Objective Multiplex PCR Design software version 2.2 (Rachlin et al. 2005).

Campylobacter primers were designed for the cytolethal distending toxin B (cdtB) gene based on the genome sequence of *C. jejuni* NCTC 11168 gi|15791399:89470-90267. The cdtB forward primer begins binding at position 156 with the sequence 5'AGTTGCGCTAGTTGGAAAAAC 3'. The reverse cdtB primer begins binding at position 665 with the sequence 5'AACTTGTAAGTGGAGCAAACC 3'. This produces a 510 basepair fragment which is unique to *C. jejuni*.

Salmonella primers were designed for the invA gene, which is highly conserved in all serotypes of *Salmonella*. Primers were designed based on the

sequence gi|16763390:3038400-3040457 of *Salmonella* Typhimurium LT2. The invA forward primer begins binding at position 43 with the sequence 5'TCACCGAAAGATAAAACCTCC 3'. The reverse invA primer begins binding at position 1977 with the sequence 5'TTCGTCATTCCATTACCTACC 3'. This produces a 1935 basepair fragment which is specific only to *Salmonella.*

Statistical Analysis

The proportion of samples where the target pathogens were detected was totaled and compared using a Chi-Square analysis. All tests of difference were at the statistical significance level $\alpha = 0.05$. Calculations were performed using the FREQ procedure in SAS statistical software (SAS Institute Inc., Cary, N.C.).

RESULTS

Salmonella was not detected in any of the 270 samples collected from the three farms in this study. *C. jejuni* was identified by PCR in 110 (40.7%) of the samples collected, using each sampling method (air filter, bootie, and sponge). The proportion of *C. jejuni* positive samples in each set of 30 samples, by sample type and flock age, is shown in Table 1.

With the use of chi square statistical analysis, the air sampling method was found to have a significantly lower ($\alpha<0.05$) proportion of *C. jejuni* detected (27.8%) than the bootie method (50%) or the sponge method (44.4%) when test results from all farm and flock ages were compared. The lowest total number of samples that *C. jejuni* was detected in for the air sampling method was found in week five.

Recovery of *C. jejuni* was similar across all ages when comparing all three sampling types. However, the proportion of samples with *C. jejuni* was significantly higher at 5 weeks (70%), than at 4 weeks (40%). There was also an increase, although not statistically significant, in *Campylobacter* from bootie samples as flock age increased (Table 1).

The proportion of *C. jejuni* positive samples in each set of 30 samples, by sample type and farm, is shown in Table 2. Farm B had a significantly higher percentage of samples where *C. jejuni* was identified (54.4%) than the other two farms. Farm B had a significantly higher proportion of positive samples from the bootie and sponge methods than Farm A or C, but not from the air sampling method (Table 2). For Farm B, the proportion of samples where *C. jejuni* was

detected was significantly higher from bootie and sponge samples than for air samples.

While the number of positive samples varied greatly for most combinations of age, farm, and sample type (Figure 1). *C. jejuni* was identified in all sample sets (n=10) for each combination of farm (3), bird age (3), and sample method (3), except for air samples from Farm C at age 3 weeks, and for air samples from farm B collected at age 5 weeks.

DISCUSSION

Campylobacter has been found on 30-100% of retail chicken carcasses in several other published survey reports (Altekruse et al. 1999, Zhao et al. 2001). *Salmonella* has a much lower incidence, being found on only 4-43% depending on which part of the country is investigated (Zhao et al. 2001). The USDA reports that about 8% of the carcasses tested from chicken slaughter plants were contaminated with *Salmonella* (FSIS 2007). *Salmonella* was not found in any of the three farms sampled in this study. The low incidence on retail chicken carcasses and post-chill carcasses make the findings of this study unsurprising. Additionally, the poultry houses sampled in this study were not pre-screened for the presence of *Salmonella*.

In this study, there were no air samples where *Salmonella* was detected, although many studies have shown *Salmonella* to be capable of survival in aerosols (Lever et al. 1996; Leach et al. 1999). McDermid et al. (1996) found *Salmonella* Typhimurium to retain 100% viability after two hours of aerosolization. The USDA recommends vaccinating breeder hens and subsequently the chicks against *Salmonella* to reduce the development of the pathogen in the guts of the chicks (FSIS 2008b). If vaccinations were employed there may have been no *Salmonella* present in the poultry houses sampled or the numbers of CFU's were too low to readily detect with the methods used in the study.

Olsen et al (2009) performed a similar study in which a total of 1800 ml of air was collected per sample, along with fecal samples collected using booties

pulled over the investigator's boots. A different type of air sampler and method of analysis was used than in this study; Olsen concluded that their air sampling method had comparable sensitivity to detect *C. jejuni* as the other sampling methods. In the present study, with only two exceptions, *C. jejuni* was found at all three poultry houses, in each of the three sampling periods, and with all three sampling methods.

With increasing age, there was an increase in positive samples with sponge sampling and bootie samples, but not with air sampling. This increase was expected, as studies have found an increase in total bacterial counts as the birds age (Vucemilo et al. 2007; Oppliger et al. 2008). Contrary to the other two sampling methods, there was a decrease in positive air samples in week five. A corresponding increase in positive samples of the other two sampling methods should have been seen when using the air sampling method. This discrepancy may have been caused by changes in humidity or a decrease in animal activity within the house (Saleh et al. 2005; Vucemilo et al. 2008). Humidity and temperature varied from house to house, from between 82˚F to 73˚F, while the relative humidity varied between 95% and 59%. High humidity or less animal activity would have produced less dust and subsequently less airborne bacteria. These would not have been factors that affected the sponge or bootie sampling methods, because these methods require physical collection of samples. The majority of the bacteria present would be picked up when wiped with a sponge or contacted with a bootie.

Sponge sampling of feed pans and water lines and bootie sampling of the house litter gave a statistically similar rate of positive samples. This shows that both methods could be used to determine whether a flock is colonized. The air sampling method showed a statistically lower percentage of positive samples and may need to be slightly altered before it can be reliably used as a sole method for determination of infection. In a previous study (unpublished), air collection directly onto selective agar plates (mCCDA and Campy-cefex) provided almost no positive samples from within the poultry houses. The use of gelatin filters with an enrichment step provided a more sensitive means of detection. Differences in ventilation within the poultry houses may have also affected the collection of air samples. If ventilation is not consistently applied throughout the study, a false negative result may be found at times when the requisite air movement in the house is not achieved (Olsen et al. 2009).

Bootie and sponge samples not only demonstrate that *Campylobacter* was present in the poultry houses, but also that the feces, litter, and/or feeders have potential to transmit these bacteria. *Campylobacter* may be protected from desiccation by fecal material and a wet environment (Ramabu et al. 2004) thus allowing the pathogen to survive outside the bird's intestinal tract long enough to be spread throughout the poultry house. Berndtson et al. (1996) concluded that the much higher concentrations of pathogens in the feces would be the most likely means of conveyance between the chickens but the amounts of airborne bacteria may be enough to cause illness in farm workers or to be passed on to other nearby poultry houses. Chickens are known to be coprophagic, meaning

at times they intentionally ingest feces (Keener et al. 2004). This could be another mode of transmittance, as feces were observed in some of the feeders during sampling.

PCR is a well-established, accurate and sensitive method of bacterial detection. It is capable of finding bacteria cells whether they are viable or non-viable. The fact that an enrichment step was involved in this study shows that not only was *Campylobacter* detected in the poultry houses, but that is was more than likely viable. Although the original samples had to be transferred into separate pathogen specific broths before analysis, the primers used for DNA amplification were designed so that they could be run under the same protocol saving a substantial amount of time and work. Environmental samples may contain inhibitors to PCR analysis, but this can usually be overcome by a simple dilution or, in this case, use of an enrichment broth (Alvarez et al. 1995).

The cdtB gene was selected for identification of *Campylobacter* because of its specificity and ubiquity within the genus. Although the cytolethal distending toxin gene is present in a variety of gram-negative bacteria, the primers used in this study were designed to be highly specific with regards to only *C. jejuni.* The cdt gene makes for a great target gene because it is essential for toxin production which is a virulence factor of *Campylobacter*. Unlike other genes selected for *C. jejuni* identification, the cdt gene is thought to be universally present in almost all *C. jejuni* (Asakura et al. 2008).

The gelatin filters used for air sampling were found to inhibit PCR identification when put directly into 100 ml of phosphate buffer solution or TE

buffer, prior to the DNA amplification step. For this reason, the filter should be used as directed by the manufacturer, either placed in 100 ml of enrichment broth or placed directly onto an agar plate, and then PCR analysis can be utilized.

The humidity level inside the poultry houses affected the gelatin filter's ease of use. As stated in the manufacturer's manual the filters should only be used in environments with up to 85% relative humidity. In this study, the filters began to soften and were difficult to remove in a high humidity environment. This did not affect the finding of positive samples though, as the high humidity (above 85% RH) was found only in the poultry house on Farm C, which had a statistically similar proportion of positive results as Farm A. In conclusion, air sampling using the gelatin filters is feasible for the detection of *C .jejuni* when coupled with another type of sampling method for verification.

Continuing research

Potential continuations of this research could be a comparison of air sampling methods using the gelatin filters placed onto selective agar plates as compared to direct quantification by use of a PCR assay. An additional study could look at the applicability of transferring environmental samples directly to PCR analysis for the simultaneous detection of multiple pathogens. Worker health implications of airborne pathogens could also be investigated using air sampling techniques. Also, an applied research project could examine the use of environmental sampling for pathogens as a pre-screening process that would

allow scheduled processing of non-pathogenic or relatively low pathogen concentration flocks to be processed early in the day to limit in-plant cross contamination in slaughter facilities. Eventually it may be possible to produce certified *Campylobacter* and/or *Salmonella* free raw chicken like certain farms in Denmark have been doing since 2002 (Krause et al. 2006).

REFERENCES

Altekruse, S., N. Stern, P. Fields and D. Swerdlow (1999). "*Campylobacter jejuni* an emerging foodborne pathogen." Emerg. Infect. Dis. **5**(1): 28-35.

Arritt, F. M., J. D. Eifert, M. D. Pierson and S. S. Sumner (2002). "Efficacy of antimicrobials against *Campylobacter jejuni* on chicken breast skin." J. Appl. Poult. Res. **11**(4): 358-366.

Asakura, M., W. Samosornsuk, A. Hinenoya, N. Misawa, K. Nishimura, A. Matsuhisa and S. Yamasaki (2008). "Development of a cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the detection and identification of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter fetus*." FEMS Immunol. Med. Mic. **52**(2): 260-266.

Berndtson, E., M. Danielsson-Tham and A. Engvall (1996). "*Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process." Int. J. Food Microbiol. **32**: 35-47.

ERS. (2008). "U.S. Broiler industry: Background statistics and information." Retrieved April 10, 2009, from http://www.ers.usda.gov/News/broilerCoverage.htm.

FSIS. (2008b). "Compliance guideline for controlling *Salmonella* in poultry." Retrieved Feb 13, 2009, from http://www.fsis.usda.gov/PDF/Compliance_Guideline_Controlling_*Salmonella*_Poultry.pdf.

FSIS (2008a). "Young Chicken Baseline Data Collection Program." Retrieved April 20, 2009, from http://www.fsis.usda.gov/PDF/Young_Chicken_Baseline_Data_Collection.pdf .

FSIS. (2007). "Quarterly progress report on *Salmonella* testing of selected raw meat and poultry products: Preliminary results, July - September 2008 " Retrieved Feb 13, 2009, from http://www.fsis.usda.gov/PDF/Q3_2008_*Salmonella*_Testing.pdf.

Krause, M., M. H. Josefsen, M. Lund, N. R. Jacobsen, L. Brorsen, M. Moos, A. Stockmarr and J. Hoorfar (2006). "Comparative, collaborative, and on-site validation of a taqman PCR method as a tool for certified production of fresh, *Campylobacter*-free chickens." Appl. Environ. Microbiol. **72**(8): 5463-5468.

Leach, S., A. Williams, A. Davies, J. Wilson, P. Marsh and T. Humphrey (1999). "Aerosol route enhances the contamination of intact eggs and muscle of experimentally infected laying hens by *Salmonella* Typhimurium dt104." FEMS Microbiol Lett. **171**(2): 203-207.

Lever, M. and A. Williams (1996). "Cross-infection of chicks by airborne transmission of *Salmonella* enteritidis pt4." Lett.Appl.Microbiol.**23**(5): 347-349.

McDermid, A. and M. Lever (1996). "Survival of *Salmonella* enteritidis pt4 and *Salmonella* Typhimurium swindon in aerosols." Lett.Appl.Microbiol. **23**(2): 107-109.

Olsen, K., M. Lund, J. Skov, L. Christensen and J. Hoorfar (2009). "Detection of *Campylobacter* in air samples for continuous real-time monitoring of *Campylobacter* colonization in broiler flocks." Appl. Environ. Microbiol. **75**(7): 2074-2078.

Oppliger, A., N. Charriere, P. Droz and T. Rinsoz (2008). "Exposure to bioaerosols in poultry houses at different stages of fattening; use of real-time PCR for airborne bacterial quantification." Ann. Occup. Hyg. **52**(5): 405-412.

Rachlin J., Ding CM., Cantor C., and Kasif S.(2005). “muPlex: A Multi-Objective Approach to Multiplex PCR Assay Design.” Nucleic Acids Res. 33(Web Server Issue):W544-W547.

Ramabu, S., N. Boxall, P. Madie and S. Fenwick (2004). "Some potential sources for transmission of *Campylobacter* jejuni to broiler chickens." Lett. Appl. Microbiol. **39**(3): 252-256.

Saleh, M., J. Seedorf and J. Hartung (2005). Influence of animal age and season on bioaerosol concentrations in a broiler house. Proc. 12th Int. Congress on Animal Hygiene, Warsaw, Poland.

Stern, N. J. and M. C. Robach (2003). "Enumeration of *Campylobacter* spp. In broiler feces and in corresponding processed carcasses." J. Food Prot. **66**: 1557-1563.

Vucemilo, M., K. Matkovic, B. Vinkovic, S. Jaksic, K. Granic and N. Mas (2007). "The effect of animal age on air pollutant concentration in a broiler house." Czech Acad. Agric.Sci. **6**(52): 170-174.

Vucemilo, M., K. Matkovic, B. Vinkovic, J. Macan, V. Varnai, L. Prester, K. Granic and T. Orct (2008). "Effect of microclimate on the airborne dust and endotoxin concentration in a broiler house." Czech J. Anim. Sci. **53**(2): 83-89.

Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. White, D. Wagner and J. Meng (2001). "Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the greater Washington, D.C. area." Appl. Environ. Microbiol. **67**(12): 5431-5436.

TABLES AND FIGURES

Flock Age	Sample Type			
(wk)	Air filter	Bootie	Sponge	Total
3	33.3%^{Aa}	43.3%^{Aa}	23.3%^{Aa}	33.3%^{A}
4	36.7%^{Aa}	46.7%^{Aa}	40.0%^{Aa}	41.1%^{A}
5	13.3%^{Aa}	60.0%^{Ab}	70.0%^{Bb}	47.8%^{A}
Total	27.8%^{a}	50.0%^{b}	44.4%^{b}	40.7%

Table 1. *C. jejuni* detection by sample type and flock age (n=30).

Significant differences ($p<0.05$) in Chi-Square statistic between rows are designated with upper case superscript letters.

Significant differences ($p<0.05$) in Chi-Square statistic between columns are designated with lower case superscript letters.

	Sample Type			
	Air filter	Bootie	Sponge	Total
Farm				
A	36.7%^{Aa}	40.0%^{Aa}	26.7%^{Aa}	34.4%^{A}
B	16.7%^{Aa}	80.0%^{Bb}	66.7%^{Bb}	54.4%^{B}
C	30.0%^{Aa}	30.0%^{Aa}	40.0%^{Aa}	33.3%^{A}
Total	27.8%^{a}	50.0%^{b}	44.4%^{b}	40.7%

Table 2. *C. jejuni* detection by sample type and farm (n=30).

Significant differences ($p<0.05$) in Chi-Square statistic between rows are designated with upper case superscript letters.

Significant differences ($p<0.05$) in Chi-Square statistic between columns are designated with lower case superscript letters.

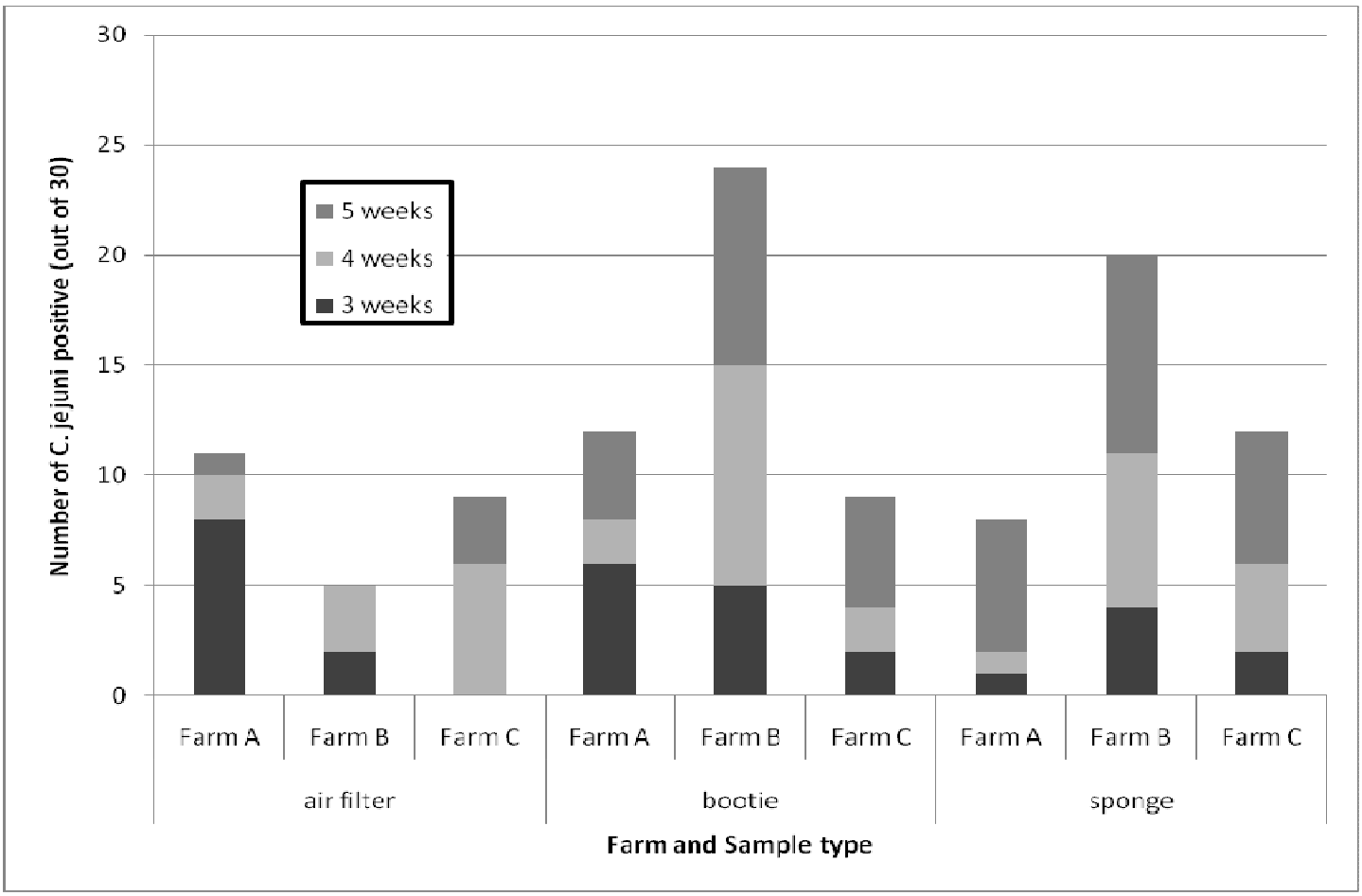

Figure 1. Total *C. jejuni* positive samples by bird age, farm, and sample method

APPENDIX

Appendix, Figure 1: Waterline with nipple water dispensers inside a poultry house

Appendix, Figure 2: Feed trays within poultry house