COMPARISON OF PLATE MEDIA FOR ISOLATION OF *CAMPYLOBACTER*

FROM LIVE BROILERS AND SCHEDULED DELIVERY OF BROILER

FLOCKS TO REDUCE CROSS-CONTAMINATION WITH

CAMPYLOBACTER

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THESIS ABSTRACT

COMPARISON OF PLATE MEDIA FOR ISOLATION OF *CAMPYLOBACTER* FROM LIVE BROILERS AND SCHEDULED DELIVERY OF BROILER FLOCKS TO REDUCE CROSS-CONTAMINATION WITH *CAMPYLOBACTER*

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Campylobacters are bacteria that are a major cause of diarrheal illness in humans and are generally regarded as the most common bacterial cause of gastroenteritis worldwide. Disease control studies have demonstrated that 50 to 70% of human *Campylobacter* illness is attributed to consuming poultry and poultry products. Broiler chickens are frequently asymptomatic carriers of *Campylobacter jejuni/coli* and the organisms are common contaminants of processed broiler carcasses. Different selective media were originally designed to isolate *C. jejuni* from human feces. However, media for isolating campylobacters from broiler samples have not been fully evaluated. The objective of our first study was to compare the efficiency of different plate media for

isolation of *Campylobacter* from broiler feces. Results from media comparison showed that mCCDA agar performed better followed by mCC, Bap-B, and Bap-H. Bap-B agar has performed similar to BAP-H agar, a version of BAP homemade in our laboratories using lysed horse blood instead of sheep blood. Campy-Line agar showed significantly lower counts when compared to other media. Colonization status of campylobacters drastically reduced on all sampling days in birds from gentamicin sulfate treated group when compared to the non-treated groups. PFGE profiles of isolates collected from different media plates showed similar pattern. The objective of our second study was to determine the effectiveness of logistic scheduling on the reduction of *Campylobacter* contamination. When, birds from positive farms with higher *Campylobacter* numbers were processed first followed by birds that have no/low contamination with *Campylobacter*, the numbers from the carcass rinse samples showed that the birds that are negative for *Campylobacter* at the farm, were positive from both pre-chill and post-chill samples. These results suggest cross-contamination of negative flocks during processing. On the contrary, negative birds processed earlier in the day, remained negative throughout processing and the positive birds that were processed following clean birds maintained a lower level of *Campylobacter* incidence. Isolates from different farms processed on the same day showed similar PFGE patterns, suggesting cross-contamination. This shows that *Campylobacter*-negative flocks can get contaminated with *Campylobacter* strains originating from the previously processed *Campylobacter*-positive flocks and that logistic scheduling may prevent cross-contamination

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I. INTRODUCTION

Campylobacter organisms have long been recognized as one of the most common bacterial causes of diarrheal illness in the United States. *Campylobacter jejuni* accounts for over 90% of the reported *Campylobacter* species from human diarrhea, with *Campylobacter coli* making up the remainder. Campylobacteriosis in humans occurs as a result of consuming contaminated milk, water and food. According to some reports, poultry meat has been found to be the most common food implicated in the spread of *Campylobacter* illness. Therefore, any effective control strategies in broiler chickens may be expected to have a considerable effect on reducing human illness. Control strategies have been improved in recent years including on-farm techniques and methods during poultry processing.

Campylobacters are microaerophilic, Gram-negative, nonspore-forming, spiral-shaped microorganisms with a unique corkscrew-like motility by means of a single polar unsheathed flagellum at one or both ends. Detection of the bacteria is usually carried out by culture in media containing selective antibiotics. As a result several methods including enrichment and plate media have been developed for the isolation of *Campylobacter* from broiler feces and commercial poultry carcasses. However, so far there is no consensus concerning the best media and methods for isolation of *Campylobacter* from live broilers.

The following literature review will discuss the efficacy of different media available for isolation of *Campylobacter* from live broilers and various reduction strategies with emphasis on logistic scheduling of commercial broiler flocks to significantly reduce the threat of logistic scheduling of commercial broiler flocks to significantly reduce the threat of *Campylobacter* contamination on broiler carcasses.

II. LITERATURE REVIEW

In 1886, Theodor Escherich described nonculturable spiral-shaped bacteria now known as campylobacters. Earlier these organisms were named Vibrio fetus by Smith and Taylor (1919), but because of their low G+C content, their microaerophilic growth requirements, and their nonfermentative metabolism, Sebald and Veron (1963) proposed a new genus *Campylobacter*. In recent years *Campylobacter spp*. are universally acknowledged as the most common bacterial cause of enteritis worldwide. In US, the Centers for Disease Control and Prevention (CDC) cited *Campylobacter* as the most common bacterial cause of diarrheal illness, with an estimated 2.4 million cases per year. Within the genus *Campylobacter, C. jejuni and C. coli* are the most common species associated with diarrheal illness in humans (Shallow et al., 1997). *C. jejuni* is implicated in about 85% of the cases of human campylobacteriosis, with the remaining cases being caused by *C. coli* (Friedman et al., 2000). The common symptoms of uncomplicated illness are fever, abdominal cramping, and diarrhea with or without blood and may last for a few days to more than 1 week. Complications such as bacteremia, endocarditis, meningitis, and urinary tract infection may also result from *Campylobacter* infections (Blaser, 1995). *C. jejuni* is now the most widely recognized antecedent cause of Guillain–Barre syndrome, an acute paralytic disease of the peripheral nervous system (Nachamkin et al., 2000). Therefore, the illness caused by these organisms are serious public health concerns.

Bacterial Characteristics

Campylobacters are Gram-negative, slender, spirally curved rods, 0.2 to 0.8 um wide and 0.5 to 5um long with a unique corkscrew like motility. Cells in old cultures may form coccoid bodies. The G+C content of the DNA ranges from 28 to 44 mol% (Nachamkin, 1995). Campylobacters are microaerophilic, grow optimally at 42^{o}C, generally produce gray, flat, irregular, spreading colonies, and are unable to oxidize or ferment carbohydrates. Campylobacters are very sensitive to dessication (Fernandez et al., 1985) and also exhibit greater sensitivity to low pH and are readily killed at pH values less than 4.9 (Blaser et al., 1980a). The organisms are oxidase and catalase positive. *C. jejuni* hydrolyzes hippurate, indoxyl acetate and reduces nitrate. The presence of hippurate hydrolase activity in *C. jejuni* is the critical biochemical reaction that distinguishes *C. jejuni* from other *Campylobacter* species.

Modes of Infection

Campylobacters can establish a permanent asymptomatic carrier state in the intestinal tract of a wide variety of wild and domestic animals, especially birds. In humans, *Campylobacter* infections are considered primarily to be the result of ingestion of contaminated foods of animal origin, although an unknown number of human illnesses may be a consequence of infections associated with pet animals or contaminated surface waters (Jacobs-Reitsma, 2000). These organisms are highly infective and cause acute gastroenteritis in humans. Infection can be induced with doses as low as 500 cells (Robinson, 1981; Black et al., 1988). The vast majority of *Campylobacter*

infections are sporadic cases and large community outbreaks are rare but have been associated with the consumption of raw milk and untreated water (Tauxe, 1992). Disease control studies have demonstrated that 50 to 70% of human *Campylobacter* illness is attributed to consuming poultry and poultry products (Tauxe 1992; Allos, 2001). Poultry accounted for at least 50% of the sporadic cases occurring among members of a health maintenance organization in Seattle (Harris et al., 1986b).

Campylobacter colonization in broilers

Broiler chickens are frequently asymptomatic carriers of *Campylobacter jejuni/coli* and the organisms are common contaminants of processed broiler carcasses. In most flocks, colonization is not detectable until at least 10 d and continues for many weeks (Newell and Wagenaar 2000). Chicks 1-2 wk old are highly susceptible to *Campylobacter* colonization (Stern 1992). Colonization is found largely in the ceca, large intestine and cloaca, and is generally restricted to the intestinal mucous layer in the crypts of the intestinal epithelium at these locations (Beery et al., 1988). It is generally accepted that colonization in chickens persists for the life span. In a positive flock, the prevalence of infection among birds is high, often reaching 100% of birds tested (Gregory et al., 1997). The spread of campylobacters among birds in a flock has been shown to occur via horizontal transmission mainly through contaminated water, litter, insects, rodents, fecal contact, and by farm personnel via their boots (Aarts et al., 1995; Evans and Sayers 2000; Line 2001). Jacobs-Reitsma (1997) reported that once a broiler in a house is found

positive for *Campylobacter*, all broilers in that house will be contaminated within 1 week, with counts remaining high until slaughter. The vertical transmission of campylobacters to flocks via contaminated eggs remains controversial. Breeder hens are usually colonized by multiple strains of *C. jejuni* (Jacobs-Reitsman 1995). Whether the presence of campylobacters in the oviduct results in the colonization of chicken embryos is unknown.

Several surveys have indicated a seasonal variation in the prevalence of poultry flock colonization. There is generally a higher rate of infection in summer than in winter. The prevalence of flock positivity is also dependent on flock size (Berndtson et al., 1996) and type of production system. Flock positivity is higher (up to100%) in organic and free-range flocks compared to intensively reared flocks (Heuer et al., 2001). Transportation of birds from farms to processing plants and holding before slaughter have also shown to increase *Campylobacter* populations (Stern et al., 1995a). *Campylobacter* consumed orally by the bird before or during crating and transportation may colonize the ceca where they may be retained throughout processing. Another source of contamination is the transport coops used to deliver birds to the processing plant. Bolder (1998) reported that excretion of pathogenic bacteria during transportation to the processing plant would cause cross contamination among birds in transport coops. Slader et al. (2002) indicated that *Campylobacter* could be isolated from transport coops even after cleaning and disinfecting and, thus the transport coops should be regarded as a potential source of *Campylobacter* contamination.

At the processing plant the potential for cross-contamination of *Campylobacter* is very high during scalding, defeathering, evisceration, washing and chilling of broiler carcasses. Poultry that enters the processing plant has *Campylobacter* populations ranging from $\log_{10}$ 5 to $\log_{10}$ 8 CFU/g of feces, and the bacteria are found in the crop as well as fecal material (Byrd et al., 1998a). With freshly slaughtered birds, campylobacters were isolated from 89% of neck skins, 93% of samples from the abdominal cavity and from 75% of samples from under the skin (Berndtson et al., 1992). *Campylobacter* has been recovered from scald water (Stern et al., 2001), which highlights that a scalding time of 2 minutes, at 60^{o}C is not enough to destroy *Campylobacter* if the initial count is high. Defeathering was reported to increase *Campylobacter* counts on carcass skins (Acuff et al., 1986; Izat et al., 1988). Berrang and Dickens (2000) assumed that contact between the rubber fingers and the abdomen of the carcass provokes some pressure that causes the release of gut content present in the lower bowel. This gut content contaminates not only the rubber fingers, but the skin of the carcasses as well.

Evisceration of carcasses is also reported to increase the number of *Campylobacter* on the carcass skin. The rupture of the crop and the intestines of birds during evisceration is a potential source of cross-contamination of the carcasses with *Campylobacter*. Byrd et al. (1998) reported that 62% of crop samples examined on the farm prior to transport to the slaughter were positive for *Campylobacter*. Following evisceration, carcasses are cooled via immersion in cold water. Lillard (1982) showed that the chill tank, in which every bird goes through, increases the risk of cross-contamination. According to Padungtod et al. (2002), during the final washing and chilling, there is a trend towards reduction of microbial counts, including *Campylobacter*

but not towards their complete elimination. This shows immersion chilling is problematic because *Campylobacter* present in some birds prior to chilling may enter the water and contaminate many other carcasses. Particularly, Waldroup et al. (1992, 1993) found that the incidence of *Campylobacter* increased from 86.4% to 90.8% of prechill and postchill carcass samples respectively. A recent study has shown that 70.7% of poultry carcasses and 91% of retail chicken products are contaminated with *Campylobacter* (Zhao et al., 2001).

Evaluation of media for isolation of *Campylobacter* from broiler samples

Different plating media can be used for isolation of *Campylobacter* from poultry samples. Selective media were originally designed to isolate *C. jejuni* from human feces by the use of cocktails of antibiotics in a rich basal medium, and by exploiting the ability of this organism to grow at 42^{o}C (Butzler and Skirrow, 1979). Typically, cefoperazone, cycloheximide, trimethoprim, rifampicin, vancomycin and polymixin B are the antibiotics used in various combinations to reduce contamination from other bacteria (Corry et al. 1995). Besides antibiotics, media for *Campylobacter* usually contain ingredients to neutralize the toxic effects of substances that form in the presence of oxygen and light. Most solid media for *Campylobacter* incorporate blood at levels between 5 and 15%, to neutralize toxic oxygen derivatives (Juven et al., 1985; Weinrich et al., 1990). Some media use defibrinated blood from sheep and others lysed horse blood. Skirrow et al. (1982) stated that lysed horse blood was needed to neutralize trimethoprim antagonists which are present in most enrichment media. A combination of ferrous sulfate, sodium metabisulphite and sodium pyruvate (FBP) was suggested by

George et al. (1978) and Hoffman et al. (1979a) to counteract the toxic effect of oxygen. Alternatives to blood or FBP include hematin and charcoal. The media most widely used today for isolation of *Campylobacter* from human feces are Skirrow, Campy-BAP, Preston, Modified charcoal cefoperazone deoxycholate agar (mCCDA), Butzler and Karmali.

Bolton and Robertson (1982) formulated Preston and CCDA media. Because, blood is expensive, has a short shelf life and is easily contaminated, they carried out a survey of alternatives to blood, and found that a combination of 0.4% charcoal, 0.25% ferrous sulfate and 0.25% sodium pyruvate allowed for a good growth of *Campylobacter* cells. Later, Hutchinson and Bolton (1984) proposed a modified medium, mCCDA, in which the antibiotic cefoperazone replaced cefazolin and the antifungal agent amphotericin B was added at a larger amount. A second selective medium called Butzler medium was proposed by Butzler and co-workers (Goossens et al., 1983). Butzler medium was said to suppress competing flora more effectively, especially Pseudomonads and Enterobacteriaceae. Karmali et al. (1986) proposed a medium, is a variation of modified CCDA, using hematin rather than ferrous sulfate; vancomycin instead of deoxycholate; and cycloheximide instead of amphotericin B. Cycloheximide was choosen because of apparent superior yeast suppression.

A medium popular with food safety microbiologists in the US for isolation of *Campylobacter* from food samples is Campy-Cefex agar (CCA; Stern et al., 1992). Several studies have reported the use of this medium for isolation of *Campylobacter* from poultry and poultry related samples. In 1999, Line proposed a new medium, Campy-Line agar (CLA), which has been shown to be highly selective. Studies analyzing broiler

carcass rinse samples showed that significantly fewer non-*Campylobacter* contaminants were observed on CLA plates.

In 1983, Bolton et al. compared Skirrow, Butzler, Campy-BAP, Blaser agar and Preston medium. Examination of *Campylobacter*-positive human feces and fecal samples from sheep, pig, cattle and chickens, indicated that no medium detected all positive samples. The order of efficiency of isolation was Preston (84%), Blaser (76%), Skirrow (74%), Campy-BAP (69%), Butzler (68%). Merino et al. (1986) tested seven plating media: Butzler, Blaser, Skirrow, Preston, mCCDA, Butzler and Preston for human diarrheal feces. In this study all the media isolated *Campylobacter* at similar frequency, but mCCDA gave the lowest number of plates with contaminating flora. Line and Berrang (2005) compared Campy-Line agar and Campy-Cefex agar for recovery of *Campylobacter* spp from poultry samples. Although Campy-Line agar showed fewer counts when compared to Campy-Cefex agar, they suggested that Campy-Line agar is easier to use due to the virtual absence of contaminating colonies. A study was conducted by Oyarzabal et al. (2005) to evaluate the different media for enumeration of *Campylobacter* from carcass rinses. It was observed that considering performance, price and preparation time, mCampy-Cefex and mCCDA produced best results when compared to CAMPY, Campy-Line, or Karmali medium. However, no statistical differences were observed. In the same study, Campy- Line agar showed lowest counts compared to other media, which was thought to be because of the large numbers of antimicrobial substances incorporated.

Intervention strategies to reduce Campylobacter on Poultry carcasses with emphasis on logistic scheduling

Historically the poultry industry has focused on strategies to reduce *Campylobacter* incidence in humans. There are a variety of approaches to reducing the incidence of pathogens in retail poultry and these can be targeted at various control points, from 'farm to fork'. Critical intervention points during the production phase have been identified, which include biosecurity in and around poultry grow-out facilities; effective wild bird, insect, and rodent control programs; litter quality, and disposal; use of closed and chlorinated watering systems; and effective hatching egg and hatchery sanitation. A significant reduction in *Campylobacter* infection in broilers after the application of strict hygienic measures has been reported by Giessen et al. (1998). However, it is generally considered that adequate biosecurity procedures are difficult to sustain in the farm environment (Pattison, 2001). In 1992, Schoeni and Doyle isolated and identified nine strains of cecum-colonizing bacteria that produced anti-*C. jejuni* metabolites. Use of these bacteria reduced the carriage of *C. jejuni* in White Leghorn chicken studies. Similarly, Aho et al. (1991, 1992) found benefit in treating chicks with specified antagonistic flora to diminish colonization by *C. jejuni*. However, Stern et al. (1988) reported that standard preparations of competitive exclusion (CE) effective against *Salmonella* apparently had no beneficial effect against colonization by *C. jejuni*. Ineffectiveness of CE was corroborated by Shanker et al. (1990), who could not detect a difference in colonization potentials of CE-treated or untreated birds. These reports suggest that CE treatment is not consistently effective against chicken colonization by *C.*

jejuni. Effective vaccine strategies directed against campylobacters in broiler chickens have yet to be developed.

At the processing plant, several procedures are suggested to minimize *Campylobacter* contamination during poultry processing. These include counter flow water systems during scalding and chilling, rinsing and washing of equipment to minimize or reduce cross-contamination, and disinfection of carcasses and related contact surfaces with chlorine or other bacterial control treatments such as trisodium phosphate or acidified sodium chlorite. A scalding temperature of 58^{o}C or above has shown to yield a significant reduction of *Campylobacter* on broiler carcasses (Izat et al., 1988). However, Berrang and Dickens (2000) found that after defeathering, *Campylobacter* counts increased significantly. It has been suggested that the rubber fingers in the mechanical picker act to cross-contaminate birds that previously had undetectable levels of *Campylobacter* (Acuff et al., 1986; Stern et al., 1995b).

Several types of carcass washers used in poultry processing plants are brush waters, cabinet washers, and inside/outside bird washers. It has been shown that washer systems currently used for inside and outside surface cleaning of chicken carcasses have limited effectiveness for *Campylobacter* removal (Bashor et al., 2004). Their study showed that although *Campylobacter* numbers were reduced by 0.5 $\log_{10}$ CFU/ml by the washers, the average concentration after the washer was still 4.3 CFU/ml of carcass rinse. All poultry processors use water chillers for rapid cooling of carcasses and to improve the shelf life of poultry products. To reduce cross contamination, the USDA (1995) required the addition of 20 to 50 ppm chlorine to chill water to prevent cross-contamination

Several antimicrobial compounds are in use as pre-chill applications or added to chill tanks to reduce microbial cross-contamination during processing. Chlorine has long been used as an antimicrobial to control microorganisms in poultry meat. Residual free chlorine is the key component with bactericidal activity. Blaser et al. (1986) showed that at a pH of 6.0, 0.1mg/L of free available chlorine in drinking water killed 99% of *C. jejuni*. Contamination of the processing equipment is reduced by increasing the chlorine concentration to 70 mg/L at pH 6.5 (Bailey et al., 1986). Acidified sodium chlorite (ASC) is another antimicrobial that is used as carcass pre-chill spray or dip solution post-chill. Ecolab (ST. Paul, Minnesota, U.S.A) markets the Sanova^{TM} spray system that is a commonly used ASC system in poultry processing plants. Kemp et al. (2001) showed a 99.2% reduction of *Campylobacter* spp. on poultry carcasses when ASC was sprayed pre-chill. It has been shown that 73.2% of carcasses were positive for *Campylobacter* after evisceration, and 49.1% were positive after the Sanova spray (Kemp et al., 2001a). Trisodium phosphate (TSP) has been approved by FSIS as an antimicrobial agent to be used on raw chilled poultry carcasses. Bashor et al., (2002) found a 1.2 $\log_{10}$ CFU reduction from broiler carcass rinse samples collected before and after TSP spray in a commercial poultry processing plant. An industrial trial by Slavik et al. (1994) demonstrated a reduction of 1.2 to 1.5 $\log_{10}$.

Waldroup et al. (1992) examined the effect on *Campylobacter* contamination by modifying several parts of the process. Their study involved a combination of six modifications to the process and was evaluated in five processing plants. The changes included use of counter-flow scalding, addition of a spray washer after scalding, use of 20

ppm of chlorine in the post-plucking washer, the water used on the evisceration-line transfer belt and in the final washer; maintenance of 1-5 ppm of free residual chlorine in the overflow to the water chilling system. This study showed a significant reduction in the levels of *C. jejuni / C. coli* in four of the five plants tested, and in the proportion of *Campylobacter* positive carcasses in two of the plants. However, it was not possible to determine the most effective of the changes made.

More research efforts are aimed at identifying clean broiler flocks before processing and at developing intervention measures during processing to avoid cross-contamination. Recently, logistic scheduling whereby flocks are slaughtered in order of contamination severity has been proposed to further reduce the risk of cross-contaminated end products. This involves processing of known *Campylobacter*-free flocks in the morning, followed by the positive flocks at the end of the day. *Campylobacter*-negative flocks can get contaminated with *Campylobacter* subtypes isolated from transport crates prior to the loading of the birds in those crates (Newell et al., 2001). Moreover, a study was conducted by Miwa et al. (2003) to determine the main source of *C. jejuni* contamination on broiler carcasses of *C. jejuni*-negative flocks during processing. The results strongly suggested that the carcasses of the *C. jejuni*-negative flocks were contaminated with *C. jejuni* strains originating from the intestines of the previously processed *C. jejuni*-positive flock. A study by Rivoal et al. (1999) reported that the same genotypes of *C. jejuni* with isolates from intestinal contents were obtained from carcasses of successive flock, suggesting cross-contamination in successive flocks. The *Campylobacter* types usually isolated from live birds are not necessarily the most

frequently types isolated from process carcasses suggesting cross-contamination of the carcasses during processing or differences in the responses by *Campylobacter* strains to the hostile environment of the plant (Slader et al., 2002). Zutter et al. (2005) applied logistic slaughter to prevent cross-contamination of carcasses with *Salmonella* and observed that logistic slaughter still allowed for carcass contamination. The results suggested that carcasses from flocks that were free of contamination on the farm were also contaminated, and in some slaughterhouses the contamination was higher for carcasses from *Salmonella*-negative flocks than from *Salmonella*-positive flocks. The aim of our studies was to determine the effectiveness of flocks scheduling on the reduction of *Campylobacter* contamination. It is logical to assume that processing *C. jejuni*-negative flocks before *C. jejuni*-positive flocks is an important tool to prevent cross-contamination of *C. jejuni*-negative carcasses of *C. jejuni*-negative flocks. However, to be applicable in practice, a successful logistic slaughter requires a very accurate and timely surveillance system for all broiler flocks. The advantages and disadvantages of such a system for commercial poultry producers will be discussed in our second experiment on logistic scheduling.

RESEARCH OBJECTIVES

Media for isolating campylobacters from broiler feces have not been fully evaluated, and there is no consensus on the best media and methods. A limited number of comparative studies have shown that no medium is entirely satisfactory for achieving an optimal rate of isolation. The purpose of our first study was to compare the performance of different plate media for isolation of artificially inoculated and naturally occurring *Campylobacter* on market-age-broiler feces. The goal of our second study was to develop a "logistic scheduling" system where flocks with lower level of *Campylobacter* contamination are processed first to avoid cross-contamination. Although *Campylobacter* contamination is reduced during processing, it has been reported that they are still present on carcass after processing at levels of 2 to 4 $\log_{10}$ organisms per gram. It has been shown that contamination of carcasses of *Campylobacter*-free flocks with *Campylobacter*-contaminated flocks may occur during processing. Therefore, logistic slaughter of known *Campylobacter*-free flocks at the beginning of the day followed by positive flocks at the end of the day may further reduce the risk of cross-contamination of the end products. Our proposal was aimed at determining if such a scheduling system applicable to the poultry production in US would allow for product that has little to no contamination to remain clean throughout processing.

III. EVALUATION OF DIFFERENT PLATE MEDIA FOR ISOLATION OF CAMPYLOBACTERS FROM LIVE BROILERS

ABSTRACT

Several selective media containing a variety of different antibiotic supplements are available for culturing thermophilic enteropathogenic campylobacters from live broilers. In the present work, we compared the efficiency of different plate media in two different experiments. In our first study eighty birds were placed in four batteries (groups A, B, C and D). The birds were challenged in drinking water with 10^5 CFU of *C. jejuni* per ml. Three birds per group were killed with CO_2 on day 8, 15, 21, 28 and 35 their ceca were removed, weighed and serially diluted in phosphate buffer solution (PBS). Birds in treatment D were gavaged with PBS containing gentamicin sulfate at different concentrations. Dilutions were plated onto BAP (Bap-B), Bap-H, modified charcoal cefoperazone deoxycholate (mCCDA), Campy-Line (CL) and modified Campy-Cefex (mCC) agar plates. In our second study, 180 fecal samples were collected in six independent trips, in which three houses from three different farms were visited per trip (total = 18 houses/farms, 10 samples per house). The samples were pooled and three pooled samples per house were tested for *Campylobacter*. Cecal samples were also collected in two out of the six trips (5 samples per house). Both fecal and cecal samples

were serially diluted in PBS and plated in triplicate on modified Campy-Cefex (mCC), modified charcoal cefoperazone deoxycholate agar (mCCDA), and Campy-Line agar CLA) plates. All plates were incubated at 42^{o}C under microaerophilic conditions for 42 h. The results obtained from media comparison from the first study showed that mCCDA agar performed better followed by mCC, Bap-B, Bap-H. However, on day 15 no statistical differences were found between media. Campy-Line agar showed fewer contaminants but significantly lower counts when compared to other media. Bap-B agar has performed similar to BAP-H agar. From experiment 2, mCC agar showed best results followed by mCCDA. Campy-Line agar plates had significantly lower counts in fecal sample plates when compared to mCC and mCCDA and very minimal or no growth on cecal sample plates. PFGE patterns differed between farms but were similar within isolates collected from the same farm. No variation was observed in PFGE patterns among isolates cultured on different media.

This study shows that direct plating technique can be used successfully for enumeration of *Campylobacter* from broiler fecal samples and mCC and mCCDA are the most economical media and produced best results compared to other media.

INTRODUCTION

Campylobacters are frequently found in the gastrointestinal tract of market-aged broilers, typically ranging from 35 to 50 days old, and as contaminants on commercially processed carcasses (Berndtson et al., 1992). Various base formulations have been used previously, to which different antimicrobial and growth supplements can be added for selectively culturing the organisms on solid media. Bolton and Robertson (1982) used nutrient broth no. 2 for their Preston media. Some media use defibrinated blood from various animals and others lysed horse blood (Skirrow et al., 1982). Blood is added to the media to neutralize toxic oxygen derivatives (Juven et al., 1985; Weinrich et al., 1990). A combination of ferrous sulfate, sodium meta bisulfite, and sodium pyruvate (FBP) was suggested by George et al. (1978) and Hoffman et al. (1979a) as an addition to *Campylobacter* media to counteract the toxic effect of oxygen derivatives. Alternatives to blood or FBP include hematin (Wesley et al., 1983; Karmali et al., 1986) and charcoal (Bolton et al., 1984; Karmali et al., 1986). A combination of antibiotics is usually added to inhibit competing bacteria present in clinical and environmental samples. Typically cefoperazone, cycloheximide, trimethoprim, rifampicin, vancomycin and polymixin B, are used in various combinations (Corry et al., 1995).

The introduction of selective agars proved successful for isolation of campylobacters from human feces and allowed for these organisms to be studied on a routine basis. The media most widely used for isolation of *Campylobacter* from human feces are those being formulated by Skirrow (1977), Butzler et al. (1983), Blaser et al. (1979), and Bolton and Robertson (1982). Studies showed that mCCDA and Karmali

agars have performed better in comparative studies using fecal samples from humans. In most of the world, the most commonly used media for analysis of poultry products is charcoal cefoperazone deoxycholate agar (CCDA, Hutchinson and Bolton, 1984). Campy-Cefex agar (CCA, Stern et al., 1992) is used primarily in the US. All the currently available media have problems with overgrowth by contaminating bacteria, which may result in failure to isolate *Campylobacter* spp. Line (1999) proposed a new medium, Campy-Line agar, that has been reported to allow fewer contaminants to proliferate. In 2005, Line and Berrang conducted a study to compare the performance of Campy-Line agar to that of Campy-Cefex agar for enumeration of *Campylobacter* from poultry feather, skin, crop, ceca and colon samples. The results suggested that Campy-Line agar has less contaminating colonies. However, Campy-Line agar showed lowest counts compared to other media from poultry carcass rinse samples (Oyarzabal et al., 2005). Currently, no single medium is universally used in laboratories for isolation of campylobacters from live broilers.

In the present study, we have evaluated different plating media for isolation of *Campylobacter* from fecal and cecal samples collected from broilers. Bap-B agar, Bap-H agar, modified charcoal cefoperazone deoxycholate (mCCDA) agar, Campy-Line (CL) agar and modified Campy-Cefex (mCC) agar have been used for comparison in two different studies from broilers. Table 3.1 shows composition of different media used.

MATERIALS AND METHODS

Sample Collection

Experiment 1: Eighty birds were placed in battery cages (2 pens/cage). Birds were housed in four groups (A, B, C and D). A standard feed treatment was given to the birds at placement. Birds were challenged with a strain of *C. jejuni* in the drinking water using phosphate buffer saline(PBS) with 10^5 CFU of *C. jejuni* per ml. This strain of *C. jejuni* has been isolated in our laboratory from retail broiler samples. The *C. jejuni* challenge started two hours after placement and lasted for 48 h. Birds were housed in four batteries (groups A, B, C and D). Three birds per group were killed with CO_2 on day 8, 15, 21, 28 and 35. Their ceca were removed, weighed and serially diluted in PBS. Dilutions were plated onto BAP (Bap-B) agar plate (Remel), a version of BAP plates done in our laboratories using lysed horse blood instead of sheep blood (Bap-H), modified charcoal cefoperazone deoxycholate agar (mCCDA, Hutchinson and Bolton 1984), Campy-Line (CL) agar plates (Line, 1999) and modified Campy-Cefex (mCC) agar plates (Oyarzabal et al., 2005). Plates were incubated for 48 h under microaerophilia (10% CO_2, 5% O_2, and 85% N_2, BOC Gases, Hixson, TN) using anaerobic jars with a MACSmics Jar Gassing System (Microbiology International, Frederick, MD). Presumptive colonies were counted and confirmed under phase contrast microscopy. Campylobacter isolates from all the plates were collected and stored at -80^{o}C, for further characterization using a multiplex polymerase chain reaction (PCR) assay and pulsed field gel electrophoresis (PFGE).

On day 5 and 6, each bird in treatment D was gavaged with PBS containing 1 mg of gentamicin sulfate per 100 g of body weight three times every 12 h. On day 27, three chickens in group D were gavaged once with PBS containing 0.7 mg of gentamicin sulfate per 100 g of body weight 12 h before being slaughtered on day 28. On day 34, three chickens in group D were gavaged once with PBS containing 1.4 mg of gentamicin sulfate per 100g of body weight 12 h before being slaughtered on day 35.

On day 4 and 15, fecal material from each group of birds was collected to determine the colonization status. Serial dilutions were made in PBS and plated on modified Campy-Cefex (mCC) plates. After 48 h of incubation under microaerophilia (10% CO_2, 5% O_2, and 85% N_2, BOC Gases, Hixson, TN; delivered to anaerobic jars with a MACSmics Jar Gassing System, Microbiology International, Frederick, MD), plates were counted and presumptive colonies confirmed under phase contrast microscopy.

On days 8, 15 and 21, three chickens from each pen were slaughtered using CO_2 and their ceca removed, weight and analyzed for *C. jejuni*. Phosphate buffer saline at a rate of 9:1 (volume:weight) was added to the ceca. Samples were stomached for 60 seconds, serially diluted and plated onto mCC plates. Plates were incubated at 42^{o}C under microaerophilia for 42 h. Presumptive colonies were confirmed under phase contrast microscopy.

Experiment 2: Fecal and cecal samples were collected from commercial broiler farms for the analysis of *Campylobacter*. Fecal samples were collected in six independent trips,

in which three houses from different farms were visited per trip (total = 18 houses/farms). Ten fecal samples were collected per house, pooled into three samples each house. Each sample consisted of 1 g of fresh fecal material weighed on site and immediately added to9 ml of Preston broth and kept under microaerophilic conditions generated with CampyGen™ (Oxoid Ltd., Basingstoke, Hampshire, England). Each pooled sample was considered a replicate for statistical purposes.

Cecal samples were also collected in two out of the six trips. Five birds were euthanized per house and their ceca removed, weighed and placed in Whirl-Pak® bags (Nasco, Fort Atkinson, WI). Preston broth was added to obtain a ratio of 1:9 (weight:volume). Samples were kept under microaerophilic conditions as described above. Each bird was considered a replicate for statistical purposes.

Both fecal and cecal samples were serially diluted in phosphate buffered saline (PBS) and plated in triplicate on mCC, mCCDA and Campy-Line agar plates. The plates were incubated at 42^{o}C for 48 h under a microaerophilic gas mixture (10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) in sealed, plastic bags (Oyarzabal et al., 2005).

Campylobacter Identification

Campylobacter colonies were considered presumptive positive if they showed the typical morphology and motility under phase contrast microscopy (Optiphot-2, Nikon, Tokyo, Japan). All presumptive isolates from the same plate and dilution were collected and individually stored at -80^{o}C in tryptic soy broth (TSB, Difco, Detroit, MI) supplemented with 30% glycerol (vol/vol) and 5% blood. Confirmation of isolates was done with

Hippurate test and multiplex PCR assay that identify *Campylobacter jejuni* and *Campylobacter coli.* All isolates were subtyped using pulsed field gel elctrophoresis (PFGE).

Hippurate test: To differentiate *Campylobacter jejuni* and *Campylobacter coli*, a rapid Hippurate Test Kit (Hardy Diagnostics, Santa Maria, CA) was used. The test employs Ninhydrin as the indicator, which detects glycine, the second byproduct of hippurate hydrolysis. The protocol provided by the manufacturer was followed. Briefly, a heavy suspension of the organism was made in the Hippurate reagent using a heavy inoculum from an 18-24 hour culture. The tubes were incubated for 2 h at 37^{o}C. After incubation, two drops of Ninhydrin indicator solution was added to each tube and the tubes were reincubated at 37^{o}C for 10-15 minutes. A positive test (*C. jejuni*) is indicated by the appearance of a deep blue color and the negative reaction (*C. coli*) by a faint blue color change or no color change

PCR Assay for identification of species: Strains were grown on mCC agar plates from stock cultures under microaerophilic conditions at 42^{o}C for 24 h. Bacterial DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA). DNA samples were also tested with a multiplex PCR assay that is based on three sets of primers targeting the aspartokinase gene (forward primer CC18F, 5'-GGT ATG ATT TCT ACA AAG CGA G-3'; reverse primer CC519R, 5'-ATA AAA GAC TAT CGT CGC GTG-3'; Linton et al., 1997), the hippuricase gene (forward primer HipO-F, 5'-GAC TTC GTG CAG ATA TGG ATG CTT-3'; reverse primer HipO-R, 5'-GCT ATA ACT ATC CGA

AGA AGC CAT CA-3') and the 16S rDNA genes (forward primer 16S-F, 5'-GGA GGC AGC AGT AGG GAA TA-3'; reverse primer 16S-R 5'-TGA CGG GCG GTG AGT ACA AG-3'). Primers for the hippuricase and 16S rDNA genes have been published by Persson and Olsen (2005). PCR assays were performed in 25 μl aliquots, with 18 μl of apre-made mix (OmniMix® HS, Cepheid, Sunnyvale, CA), 1 μl of each primer (10 μM) and 1 μl of the DNA template (~50 ng). The assay was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). A temperature of 94°C for 4 min was used to denature DNA, followed by 20 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. Final extension was done at 72°C for 5 min. Amplicons were detected by standard gel electrophoresis in 1.5% agarose (Ultra Pure DNA Grade Agarose, Bio-Rad Laboratories, Hercules, CA, USA) in Tris–borate–EDTA buffer at 70 V for 1 h. Gels were stained with ethidium bromide. DNA bands in the gels were stained with ethidium bromide and visualized using a UV transilluminator (Gel-Doc System) with a Molecular Analyst computer program (Bio-Rad Laboratories).

PFGE Analyses**:** The characterization of the isolates was established by pulsed field gel electrophoreses (PFGE) analyses with the restriction endonuclease *SmaI*. We followed a described pulsed-field gel electrophoresis protocol (Ribot et al., 2001; http://www.cdc.gov/pulsenet/protocols.htm). Pair comparison and cluster analysis was done using the Dice correlation coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm of BioNumerics version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 1%. A

cutoff of 90% was used for the determination of the different PFGE patterns (De Boer et al., 2000)

Statistical Analyses

Bacterial counts were converted to $\log_{10}$ CFU/g of fecal/cecal material. Means were analyzed for differences using Duncan's test (GLM procedure of SAS), and the standard error calculated with Proc Means (SAS Institute Incorporated, Cary, NC). For all tests, a *P* value of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

The numbers of *Campylobacter* recovered per gram of fecal/cecal sample type from the battery house are shown in Tables 2 through 4 (Experiment 1). Results are expressed as $\log_{10}$ mean counts. Table 2 shows the colonization status of *Campylobacter* in broilers on day 4 and day 15, following challenge with *C. jejuni*. Results from media comparison showed that mCCDA agar performed better followed by mCC, Bap-B, Bap-H. However, on day 15 no statistical differences were found between media (Table 3). The differences among media were more consistent on day 35 (Table 3), when birds are nearing market-age, which is also a better time for collecting samples to assess the most suitable medium for direct isolation of *Campylobacter*. Our findings suggest that charcoal-based media are as effective as blood-containing media for isolating *Campylobacter* spp. from cecal samples. Campy-Line agar showed significantly lower counts when compared to other media except on day 15 (Tables 3). The large numbers of antimicrobial substances

incorporated in this medium may have resulted in high selectivity (Oyarzabal et al.,2005). This shows that antibiotics added to increase selectivity of a medium may harm recovery of the target organism also, and thus result in recovery of fewer *Campylobacter* than the other less selective media. Bap-B agar has performed similar to BAP-H agar aversion of BAP homemade in our laboratories using lysed horse blood instead of sheep blood. By replacing pre-made agar (Bap-B) with homemade agar (Bap-H), the cost of the medium can be reduced considerably without affecting its efficacy for the recovery of *Campylobacter* spp.

The results from Table 4 show variability in *Campylobacter* colonization status of birds from different groups. This variability can be attributed to the variable growth of *Campylobacter* on different media and the methodology used. Figure 1 shows the PFGE pattern of the control C. *jejuni* strain, the isolates collected on days 8, 15, 21, 28 and 35 and isolates collected from different media on day 35. No variable patterns were observed indicating that these strains were not different from the control strain.

Antibiotics may be indicated in patients with a more severe or prolonged *Campylobacter* illness and there is some evidence that erythromycin may produce a rapid clinical and bacteriological cure (Butzler et al., 1979; Karmali et al., 1979). Potential altematives to erythromycin in the treatment of *Campylobacter* enteritis include tetracycline, doxycycline, gentamicin, nitrofuran derivatives, and chloramphenicol. Parenteral gentamicin has been successfully used to treat *C. jejuni* septicemia in humans (Butzler et al., 1979). In a study conducted by Karmali et al. (1981), over 90% of *Campylobacter* strains were inhibited by gentamicin at concentrations of 1.0 μg/ml or less. In our study, the birds were gavaged with gentamicin sulfate on days 5, 6, 27 and 34.

The birds were slaughtered on days 8, 28, and 35 to determine the colonization status of *Campylobacter* following treatment with gentamicin. The results showed that colonization status of campylobacters was drastically reduced on all sampling days in birds from group D treated with gentamicin sulfate when compared to the non-treated groups (Table 4). The birds in group D were completely negative (no growth seen on plates) for *Campylobacter* on day 8 but not on days 28 and 35, possibly because the concentration of gentamicin sulfate on respective days is not sufficient to completely inhibit all of the *Campylobacter* colonization at that stage, which means that it depends on the colonization status of the bird and or the resistance of the campylobacters to gentamicin. It is also observed that a lower number of campylobacters have been recovered from different plate media on days 8, 28 and 35 when compared to those on days 15 and 21 (Table 3), which may be attributed to the inhibitory effect of gentamicin.

The results from experiment 2 are from commercial broiler farm samples. Of the three media compared to recover naturally occuring *Campylobacter* from broiler samples, mCC agar showed best results followed by mCCDA. Once again, Campy-Line agar plates showed no contamination from other bacteria. However, these plates had significantly lower counts in fecal sample plates when compared to mCC and mCCDA and very minimal or no growth on cecal sample plates. (Tables 5 & 6). *Campylobacter* colonization status of the birds from positive farms ranged from 1.1 $\log_{10}$ CFU/g to 6.9 $\log_{10}$ CFU/g (Table 7). This shows that birds can harbor very high levels of *Campylobacter* up to 6.9 $\log_{10}$ CFU/g, and thus poultry meat can be a potential source of contamination to humans. No significant variation is observed among farms in *Campylobacter* numbers from cecal samples (results not shown). Hippurate test and

multiplex PCR have identified the majority of species to be *C. jejuni*. *C. coli* was isolated from both fecal and cecal samples from one of the farms. Also, few samples showed a mixed culture (C. *jejuni* and *C. coli).* PFGE patterns differed between farms but were similar within isolates collected from the same farm (Figure 2). No variation was observed in PFGE patterns among isolates cultured on different media with an exception (Figure 3).

Our results show that direct plating can be used successfully for isolation of *Campylobacter* from broiler samples. The medium chosen may affect the recovery of *Campylobacter* spp. Considering performance and cost, mCC and mCCDA would be the media of choice for isolation of *Campylobacter* from broiler fecal and cecal samples.

Table 3.1: Composition of different media used in the experiment

Medium	Base	Supplement
Campy-Cefex	Brucella agar, ferrous sulfate sodium bisulfite, sodium pyruvate deionized water	Laked horse blood, cefoperazone cycloheximide
mCampy-Cefex	Brucella agar, ferrous sulfate sodium bisulfite, sodium pyruvate deionized water	Lysed horse blood, cefoperazone
mCCDA	Nutrient broth no. 2, bacteriological charcoal, casein hydrolysate, sodium desoxycholate, ferrous sulfate, sodium pyruvate, agar, deionized water	Cefoperazone, amphotericin B
Campy-Line	Brucella agar, ferrous sulfate, sodium bisulfite, sodium pyruvate, alpha-keto-glutaric acid, sodium carbonate, deionized water	Hemin, polymyxin B sulfate trimethoprim, vancomycin, cycloheximide, cefoperazone, triphenyl tetrazolium chloride
Bap-B	Brucella agar, ferrous sulfate, sodium-bisulfite, sodium pyruvate, deionized water	Sheep blood, vancomycin, polymyxin trimethoprim, cephalothin, amphotericin B
Bap-H	Brucella agar, ferrous sulfate, sodium-bisulfite, sodium pyruvate, deionized water	Lysed horse blood, vancomycin, polymyxin B, trimethoprim, cephalothin amphotericin B

Table 3.2. Recovery of *Campylobacter* spp. from broiler feces following challenge with *C. jejuni*

	Log CFU/g fecal material	
Group	Day 4	Day 15
A	9.0	7.5
B	9.3	7.0
C	9.5	6.7
D	10.0	7.0

Table 3.3. Recovery of *Campylobacter* spp. from different plate media by sampling days (Log CFU/g ceca)

Day	Plate	Mean	Std Error
8	Bap-B	5.5 ^{A,B}	0.83
	Bap-H	5.3 ^{A,B}	0.78
	mCCDA	5.8 ^{A}	0.91
	CL	4.8 ^{B}	0.83
	mCC	5.7 ^{A,B}	0.84
15	Bap-B	7.9 ^{A}	0.25
	Bap-H	7.5 ^{A}	0.26
	CCDA	7.9 ^{A}	0.26
	Line	7.5 ^{A}	0.18
	mCC	8.0 ^{A}	0.34
21	Bap-B	8.1 ^{A,B}	0.15
	Bap-H	7.8 ^{B,C}	0.21
	mCCDA	8.3 ^{A}	0.17
	CL	7.5 ^{C}	0.21
	mCC	8.1 ^{A,B}	0.17
28	Bap-B	7.6 ^{A,B}	0.40
	Bap-H	7.4 ^{A,B,C}	0.40
	mCCDA	7.7 ^{A}	0.39
	CL	6.9 ^{C}	0.42

	mCC	7.2 [B,C]	0.65
35	Bap-B	7.8 [A]	0.42
	Bap-H	7.6 [A]	0.37
	mCCDA	8.1 [A]	0.34
	CL	6.0 [B]	0.39
	mCC	7.6 [A]	0.42

Table 3.4. Recovery of *Campylobacter* spp. from broiler cecal samples from different groups by sampling days (Lof CFU/g ceca)

Day	Group	Mean	Std Error	Ceca Weight	Std Error
8	A	6.6 ^{B}	0.22	1.7 $^{A, B}$	0.07
	B	7.2 ^{A}	0.36	1.5 ^{B}	0.07
	C	7.6 ^{A}	0.19	1.8 ^{A}	0.09
	D[1]	1.0 ^{C}	0	1.5 ^{B}	0.14
15	A	7.2 ^{C}	0.18	3.4 ^{A}	0.86
	B	8.8 ^{A}	0.20	3.2 ^{A}	0.75
	C	8.2 ^{B}	0.26	5.2 ^{A}	1.07
	D	7.1 ^{C}	0.06	3.5 ^{A}	0.72
21	A	7.9 ^{B,C}	0.14	7.2 ^{A}	0.51
	B	7.6 ^{C}	0.16	8.1 ^{A}	1.27
	C	8.5 ^{A}	0.13	6.5 ^{A}	1.03
	D	8.2 ^{A,B}	0.20	7.5 ^{A}	2.66
28	A	8.3 ^{A}	0.15	10.2 ^{A}	2.93
	B	8.5 ^{A}	0.11	6.4 ^{A}	0.34
	C	8.1 ^{A}	0.15	6.4 ^{A}	0.83
	D[2]	5.0 ^{B}	0.30	9.0 ^{A}	1.79

35	A	8.6 [A]	0.08	11.7 A	1.43
	B	8.7 [A]	0.08	9.1 A	2.26
	C	8.0 [B]	0.14	11.1 A	0.82
	D	5.9 [C]	0.35	10.5 A	2.07

[1] Negative at 10^3. given a value of 1 log CFU/g

[2] Negative at 10^4. given a value of 3 log CFU/g

Day 28 mCC was not good, contaminated. Two samples in D were negative at 10^4; we assigned 3 log to each.

Table 3.5. Comparison of three different media for recovery of *Campylobacter* spp. from broiler feces from commercial farms

Plate	Mean	Std Error
mCC	5.8 [A]	0.20
mCCDA	4.0 [B]	0.37
CL	2.4 [C]	0.49

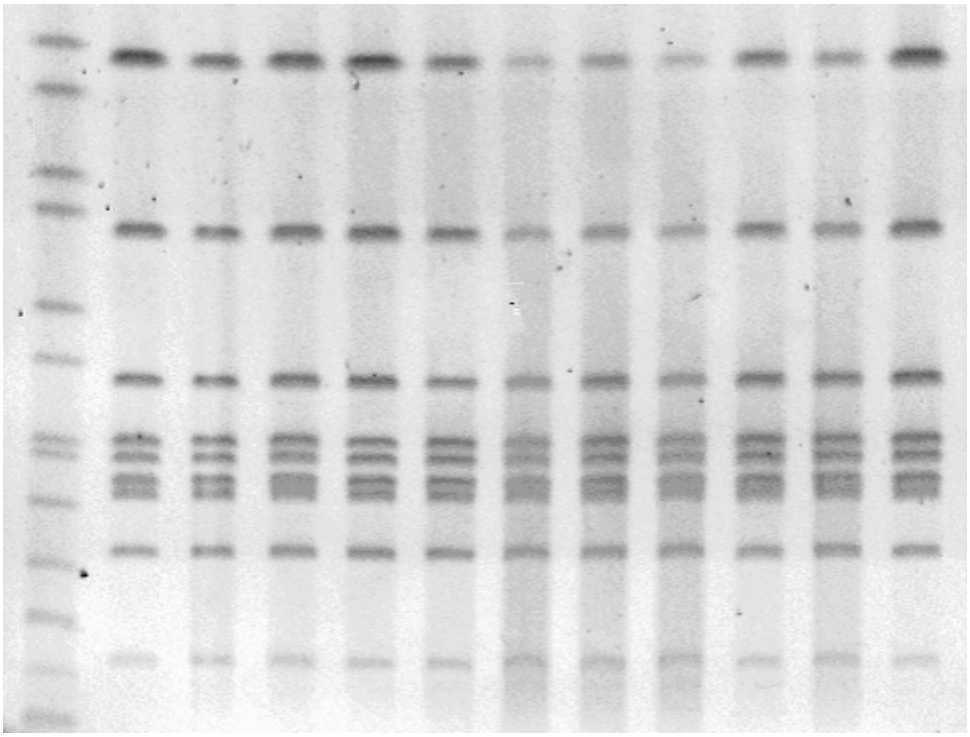
Table 3.6. Comparison of three different media for recovery of *Campylobacter* spp. from broiler cecal samples from commercial farms

Plate	Mean	Std Error
mCC	4.5 [A]	0.20
mCCDA	4.1 [A]	0.55
CL	0.1 [B]	0.13

Table 3.7. Recovery of *Campylobacter* spp. from broiler feces from different farms

Farm	Observations	Mean	Std Error
F6	9	6.9 ^{A}	0.22
F11	9	6 ^{A,B}	0.48
F10	9	5.7 ^{A,B,C}	0.37
F7	9	5.3 ^{A,B,C}	0.17
F9	9	4.7 ^{A,B,C,D}	0.27
F13	9	4.2 ^{B,C,D}	0.61
F8	9	3.8 ^{B,C,D}	0.97

Figure 3.1. PFGE profiles of selected isolates from experiment 1



L A1 A2 A3 A4 A5 A6 C M BB BH CL

L = Ladder (50 kbp)

A1 = *C. jejuni* control

A2-A6 = *Campylobacter* isolates collected on days 8,15,21,28 and 35

C = mCCDA

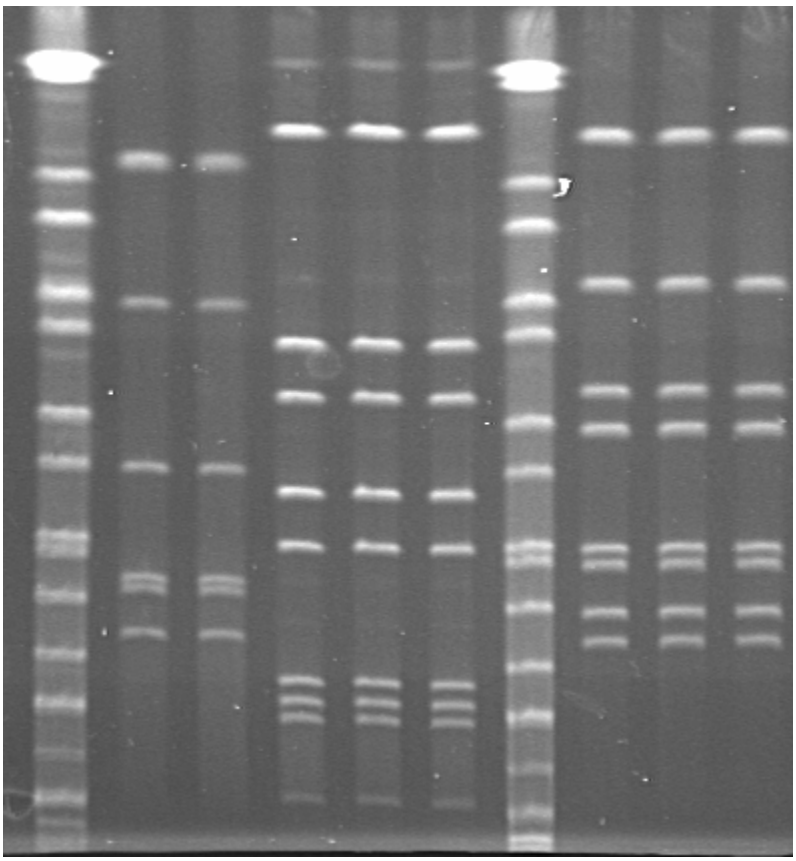
M = mCC

BB = Bap

BH = Bap-H

CL = Campy-Line

Figure 3.2. PFGE profiles of selected isolates from experiment 2



C1 508M 508C 526M 526C 526L C2 529M 529C 529L

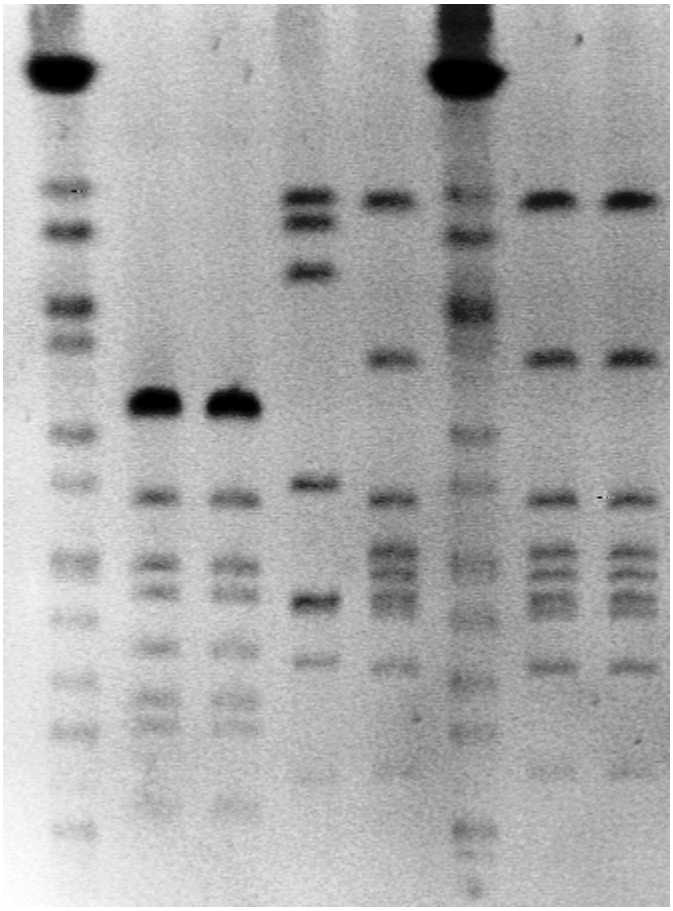
C1, C2 = S.enteritidis control

M = mCC

C = mCCDA

L = Campy-Line

Figure 3.3. PFGE profiles of selected isolates from experiment 2



C1 547M 547C 550M 550C C2 553M 553C

C1, C2 = S.enteritidis control

M = mCC

C = mCCDA

IV. LOGISTIC SCHEDULING OF COMMERCIAL BROILER FLOCKS TO REDUCE CROSS-CONTAMINATION WITH *CAMPYLOBACTER* DURING PROCESSING

ABSTRACT

Logistic scheduling whereby flocks are slaughtered in order of contamination severity has been evaluated to reduce the risk of *Campylobacter* cross-contamination on broiler carcasses. Broiler fecal samples were collected in 7 individual trips to commercial broiler farms. Ten fecal samples per flock were collected (total=3 farms/visit; 30 samples/visit), weighed (1 g each) and mixed with 9 ml of Preston broth. The samples were serially diluted in PBS. According to the level of *Campylobacter* contamination, flocks were divided as negative/low contamination, and high contamination flocks. A simple scheduling of processing was designed in which negative/low flocks were processed first to avoid cross-contamination or processing the flocks with the highest *Campylobacter* contamination first to induce cross-contamination. At the processing plant, each flock was sampled for *Campylobacter* spp. with the carcass rinse technique before the chiller and after chiller. The samples were plated onto modified Campy-Cefex agar and modified charcoal cefoperazone deoxycholate agar plates and incubated at 42^{o}C for 48 h. The results suggested that *Campylobacter*-negative flocks processed after the

Campylobacter- positive flocks have been contaminated with *Campylobacter* strains originating from the previously processed *Campylobacter*-positive flocks. Few carcass rinse isolates from different farms processed on the same day showed similar PFGE patterns, suggesting cross-contamination.

INTRODUCTION

Campylobacteriosis is one of the most common causes of diarrheal diseases in humans worldwide (Altekruse et al., 1999). Broiler chickens are frequently asymptomatic intestinal carriers of *Campylobacter jejuni/coli* and the organisms are common contaminants of processed broiler carcasses (Stern et al., 2001). Therefore, mishandling of raw poultry and consumption of undercooked poultry can be important risk factors for human campylobacteriosis. There is considerable interest in reducing *Campylobacter* infection in poultry production and diminishing levels of contamination on processed carcasses. According to Bryan and Doyle (1995) poultry slaughterhouse is a principal source of *Campylobacter* contamination of raw meat. A considerable part of live broiler chicken flocks that are received in processing plants are positive for *Campylobacter* (Mead et al., 1995). Cross contamination of carcasses between different flocks occurs during processing (Rivoal et al., 1999; Newell et al., 2001). Potential sources of *Campylobacter* contamination on poultry carcasses include fecal contamination of feathers and skin during transport to the processing plant, leakage of fecal contents from the cloaca, intestinal breakage and contact with contaminated equipment, water or other carcasses during processing (Jacobs-Reitsma, 2000). Several authors reported that cross-contamination of *Campylobacter* is very high during scalding, defeathering, evisceration, washing and chilling of broiler carcasses. However, the main sources of carcass contamination during processing have not been fully clarified.

Miwa et al. (2003) conducted a study to determine the source of *C. jejuni* contamination on broiler carcasses. The results suggested that the most important source

of carcass contamination of *Campylobacter*-negative flocks is the intestinal content of previously processed *Campylobacter*-positive flocks. Rivoal et al. (1999) reported that the same genotypes of *C. jejuni* with isolates from intestinal contents were obtained from carcasses of successive flocks, and suggested cross-contamination in successive flocks. Therefore, logistic scheduling which involves processing of known *Campylobacter*-free flocks in the morning, followed by the positive flocks at the end of the day may be expected to reduce cross-contamination of broiler carcasses.

The objective of our study was 1) to determine the colonization status of the flocks before slaughter and develop a scheduling system where flocks with lower levels of *Campylobacter* contamination are processed earlier on the processing day to avoid cross-contamination and 2) to identify the *Campylobacter* isolates obtained, to the species level and subtyping to determine correlations between isolates from the farm and the plant.

Materials and Methods

Sample collection from farms

Broiler fecal samples were collected in 7 individual trips to commercial broiler farms. At each visit, three flocks scheduled to be processed on the same day and at the same processing plant are tested for the level of *Campylobacter* colonization a week before processing. Ten fecal samples per flock were collected (total=3farms/visit 30samples/visit), weighed (1 g each) and mixed with 9 ml of Preston broth. The samples were serially diluted in PBS and plated onto modified Campy-Cefex (Oyarzabal et al., 2005) agar and modified charcoal cefoperazone deoxycholate (mCCDA, Hutchinson and Bolton 1984) agar plates in triplicate. The plates were incubated at 42^{o}C for 48 h under a microaerophilic gas mixture (10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) in sealed, plastic bags (Oyarzabal et al., 2005).

Sample collection from processing plant

Ten pre-chill and ten post-chill carcass rinses were collected per visit from the farms processed according to the scheduling system. Samples were collected using the carcass rinse method (Pathogen reduction, HACCP final rule. USDA, 1996). Each carcass rinse was plated on to modified Campy-Cefex (Oyarzabal et al., 2005) agar and modified charcoal cefoperazone deoxycholate (mCCDA, Hutchinson and Bolton 1984) agar plates. For each medium, two plates were each spread with 0.1 ml of the carcass rinse and four plates were each spread with 0.25 ml of the rinse. The plates were incubated at 42^{o}C for 48 h under a microaerophilic gas mixture (10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) in sealed, plastic bags (Oyarzabal et al., 2005)

Campylobacter Identification

Typical *Campylobacter* colonies were considered presumptive positive if they showed the typical morphology and motility under phase contrast microscopy (Optiphot-2, Nikon, Tokyo, Japan). All isolates from the farms and processing plants were collected and individually stored at -80^{o}C in tryptic soy broth (TSB, Difco, Detroit, MI) supplemented with 30% glycerol (vol/vol) and 5% blood. Confirmation of isolates was done with hippurate test and a multiplex PCR assay that identify *Campylobacter jejuni* and *Campylobacter coli.* All isolates were subtyped using pulsed field gel electrophoresis (PFGE).

Hippurate test: To differentiate *Campylobacter jejuni* and *Campylobacter coli*, a rapid Hippurate Test Kit (Hardy Diagnostics, Santa Maria, CA) was used. The test employs Ninhydrin as the indicator, which detects glycine, the second byproduct of hippurate hydrolysis. The protocol provided by the manufacturer was followed. Briefly, a heavy suspension of the organism was made in the Hippurate reagent using a heavy inoculum from an 18-24 hour culture. The tubes were incubated for 2 h at 37^{0}C. After incubation, two drops of Ninhydrin indicator solution was added to the tube and the tubes were reincubated at 37^{0}C for 10-15 minutes. A positive test (*C. jejuni*) is indicated by the appearance of a deep blue color and the negative reaction (*C. coli*) by a faint blue color change or no color change.

PCR Assay for identification of species: Strains were grown on mCC agar plates from stock cultures under microaerophilic conditions at 42^{o}C for 24 h. Bacterial DNA was

extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA). DNA samples were also tested with a multiplex PCR assay that is based on three sets of primers targeting the aspartokinase gene (forward primer CC18F, 5'-GGT ATG ATT TCT ACA AAG CGA G-3'; reverse primer CC519R, 5'-ATA AAA GAC TAT CGT CGC GTG-3'; Linton et al., 1997), the hippuricase gene (forward primer HipO-F, 5'-GAC TTC GTG CAG ATA TGG ATG CTT-3'; reverse primer HipO-R, 5'-GCT ATA ACT ATC CGA AGA AGC CAT CA-3') and the 16S rDNA genes (forward primer 16S-F, 5'-GGA GGC AGC AGT AGG GAA TA-3'; reverse primer 16S-R 5'-TGA CGG GCG GTG AGT ACA AG-3'). Primers for the hippuricase and 16S rDNA genes have been published by Persson and Olsen (2005). PCR assays were performed in 25 μl aliquots, with 18 μl of a pre-made mix (OmniMix® HS, Cepheid, Sunnyvale, CA), 1 μl of each primer (10 μM) and 1 μl of the DNA template (~50 ng). The assay was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). A temperature of 94^{o}C for 4 min

was used to denature DNA, followed by 20 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. Final extension was done at 72°C for 5 min. Amplicons were detected by standard gel electrophoresis in 1.5% agarose (Ultra Pure DNA Grade Agarose, Bio-Rad Laboratories, Hercules, CA, USA) in Tris–borate–EDTA buffer at 70 V for 1 h. Gels were stained with ethidium bromide. DNA bands in the gels were stained with ethidium bromide and visualized using a UV transilluminator (Gel-Doc System) with a Molecular Analyst computer program (Bio-Rad Laboratories).

PFGE Analyses: The characterization of the isolates was established by pulsed field gel electrophoreses (PFGE) analyses with the restriction endonuclease *SmaI*. We followed a described pulsed-field gel electrophoresis protocol (Ribot et al., 2001; http://www.cdc.gov/pulsenet/protocols.htm). Pair comparison and cluster analysis was done using the Dice correlation coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm of BioNumerics version 4.50 (Applied Maths, Austin, TX). The optimization and position tolerance for band analysis were set at 1%. A cutoff of 90% was used for the determination of the different PFGE patterns (De Boer et al., 2000).

Statistical Analysis

Campylobacter counts were converted to $\log_{10}$ CFU/g of fecal material. Means were analyzed for differences ($P \leq 0.05$) using Duncan's test (GLM procedure of SAS), and the standard error calculated with Proc Means (SAS Institute Incorporated, Cary, NC).

RESULTS AND DISCUSSION

Out of 21 flocks sampled, 12 flocks were positive for *Campylobacter* at the farm level (Table 1). In the *Campylobacter*-positive flocks, 100% (10/10) of the birds examined were positive. In trials I, II, and VI, birds from positive farms with higher *Campylobacter* numbers were processed first followed by birds that have no/low contamination with *Campylobacter*. The numbers from the carcass rinse samples from these trials show that the birds that are negative for *Campylobacter* at the farm, were positive from both pre-chill and post-chill samples. These results suggest cross-contamination of negative flocks during processing. On the contrary, in trials III, IV and VII, birds from *Campylobacter*-negative farms were processed first. The results show that negative birds remained negative throughout processing and the positive birds that were processed following clean birds, maintained a lower level of *Campylobacter* incidence. The numbers from post-chilled carcass rinse samples were significantly lower than those obtained from live birds. These results suggest that logistic scheduling may reduce the levels of *Campylobacter* during processing. In trial V, all 3 farms tested were positive for *Campylobacter* while at the farm, and during processing flock 14 that had low incidence of *Campylobacter* compared to flocks 13 and 15, was processed first right after clean up. As a result, the pre-chill and post-chill samples from flock 14 were negative for *Campylobacter* and those from flock 13 showed significant reduction in *Campylobacter* numbers at the post-chill level (6.0 $\log_{10}$ CFU/g at the farm to 0.5 $\log_{10}$ CFU/ml post-chill).

The results suggest that *Campylobacter*-negative flocks processed after the *Campylobacter*-positive flocks may have been contaminated with *Campylobacter* strains

originating from the previously processed *Campylobacter*-positive flocks. We have performed multiplex PCR and PFGE on all isolates, to compare the genetic profiles and determine correlations between the isolates collected from the farms and isolates from the processing plant samples. Figure 1 shows PFGE profiles of isolates from flocks 4, 5 and 6. In this trial, (II) flock 6 which was *Campylobacter*-positive was processed first, followed by flocks 4 and 5 which were *Campylobacter*-negative. All the isolates from the carcass rinse samples show similar pattern, suggesting that the *Campylobacter* strain from flock 6 may have contaminated carcasses from flocks 4 and 5. Figure 2 shows correlation between isolates from farm and processing plant samples from flock 12. This flock was processed after a *Campylobacter*-negative flock in Trial IV. From the similar

PFGE pattern the isolates from the carcass rinses can be linked to the isolates from the farm which explains that the *Campylobacter* strain found on carcasses is the same that has been carried by the birds from the farm. The PFGE profiles from flocks 13, 14 and 15 are shown in Figure 3. All 3 flocks were positive for *Campylobacter* at the farm. The PFGE pattern of isolates collected from live birds of flock 14 was also observed in a few isolates from carcass rinses from successive flocks 13 and 15. This suggests that cross-contamination may have occurred in successive flocks.

Carcass contamination of *Campylobacter* during processing ultimately results in the contamination of end products posing a serious hazard for public health. Our study shows that *Campylobacter*-negative flocks can get contaminated with *Campylobacter* strains from previously processed *Campylobacter*-positive flocks and that logistic scheduling may prevent cross-contamination. This type of scheduling also helps positive flocks to significantly reduce levels of *Campylobacter* on the carcasses by the end of processing. Therefore, in addition to efficient cleaning and disinfection, and implementing various reduction strategies, logistic scheduling can also be employed by processing plants to prevent contamination pressure from *Campylobacter*-positive flocks.

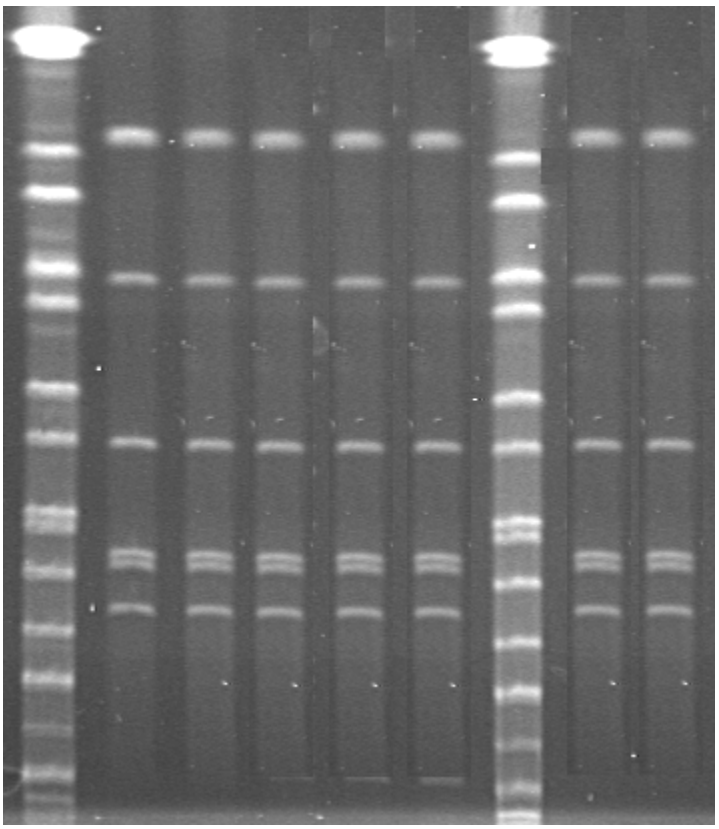
Table 4.1: Recovery of *Campylobacter* from broiler fecal (log_{10} CFU/g) and carcass rinse samples (log_{10} CFU/ml)

Farms	Live	Pre-chill	Post-chill
I			
F1	$5.8^{A,a}$	$2.2^{A,b}$	$1.4^{A,c}$
F2	N	$1.7^{A,a}$	$1.1^{A,a}$
F3	N	$2.1^{A,a}$	$0.6^{B,b}$
II			
F6	$6.3^{A,a}$	$1.9^{A,b}$	$1.0^{A,c}$
F4	N	$1.8^{A,a}$	$0.9^{A,b}$
F5	N	$1.6^{A,a}$	$0.8^{A,b}$
III			
F8	N	N	N
F7	$3.6^{B,a}$	$2.0^{A,b}$	$1.45^{A,c}$
F9	$5.2^{A,a}$	$0.9^{B,b}$	-
IV			
F10	N	N	N
F12	$6.0^{B,a}$	$2.4^{A,b}$	$0.5^{A,c}$
F11	7.6^{A}	-	-
V			
F14	5.3^{C}	N	N
F13	$6.0^{B,a}$	$2.9^{A,b}$	$0.5^{A,c}$
F15	$6.5^{A,a}$	$2.3^{B,b}$	-

VI			
F18	$6.9^{A,a}$	$2.3^{A,b}$	$0.6^{A,c}$
F16	N	$0.9^{B,a}$	$0.2^{A,b}$
F17	N	-	-
VII			
F21	N	N	N
F20	$5.7^{A,a}$	$0.7^{A,b}$	N
F19	4.8^{B}	-	-

F= flock; N= negative for *Campylobacter*; I- VII: number of trials
$^{A, B, C}$: differences in means between farms
$^{a, b, c}$: differences in means within the same farm

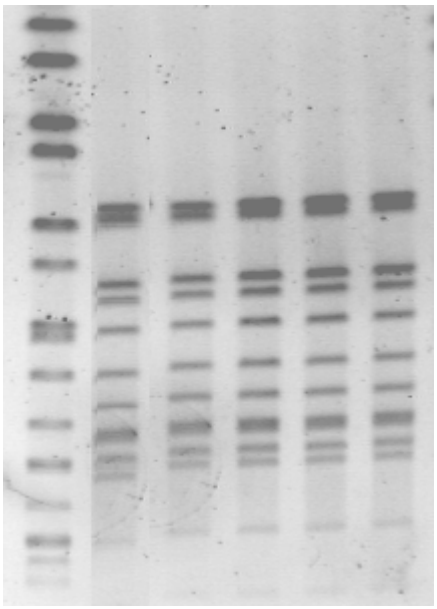
Figure 4.1: PFGE profiles of *Campylobacter* isolates from flocks 4, 5 and 6



C f6l f6pr f6po f4pr f4po C f5pr f5po

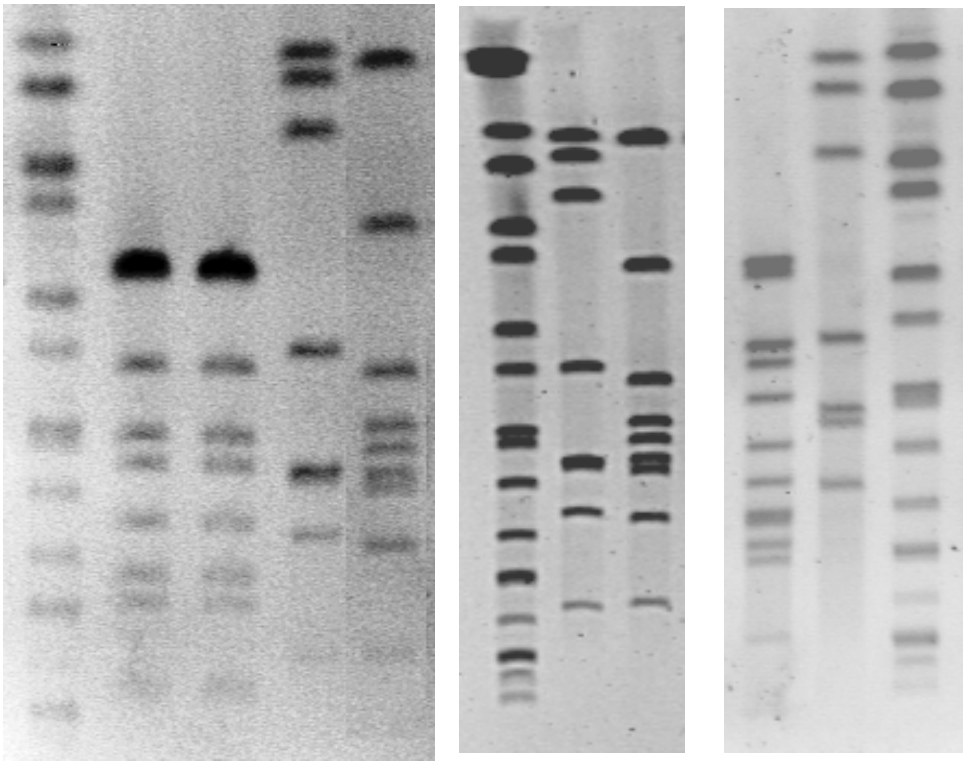
C= *Salmonella* serotype Enteritidis; f= flock; l= live birds;
pr= pre-chill; po=post-chill

Figure 4.2: PFGE profiles of *Campylobacter* isolates from flock 12



C= *Salmonella* serotype Enteritidis; l= live birds
pe= pre-chill; po= post-chill

Figure 4.3: PFGE profiles of *Campylobacter* isolates from flocks 13, 14 and 15 taken from different gels for comparison



C f13l1 f13l2 f14l1 f15l1 C f15pr1 f15pr2 f14pr f14po C

C= *Salmonella* serotype Enteritidis; f= flock; l= live birds;
pr= pre-chill; po=post-chill

SUMMARY

Our study highlighted the differences in plating media for isolation of *Campylobacter* from broiler feces. Of the plating media used, mCC and mCCDA have performed best compared to Campy-Line, Bap-B and Bap-H. Campy-Line agar recovered fewer *Campylobacter* than the other media. This shows that large number of antibiotics added to increase selectivity of a medium may harm recovery of the target organism also, and thus result in isolation of fewer *Campylobacter* than the other less selective media. Bap-B agar has performed similar to BAP-H agar a version of BAP homemade in our laboratories using lysed horse blood instead of sheep blood. By replacing pre-made agar (Bap-B) with homemade agar (Bap-H), the cost of the medium can be reduced considerably without affecting its efficacy for the recovery of *Campylobacter* spp. The performance of a particular medium will be influenced by factors such as sample type, associated competing bacteria, and the selectivity of the medium. Our findings help in choosing appropriate medium for enhanced recovery of *Campylobacter* from broiler fecal and cecal samples, and in research on intervention methods for reducing contamination of poultry products.

We have hypothesized that logistic slaughter may help reduce the health risk to consumers, of *Campylobacter* spp. on chicken meat. Our study to test the effectiveness and efficiency of logistic slaughter showed that testing all broiler flocks and processing all positively tested flocks either at the end of the day or on a different processing line

reduces cross-contamination of broiler carcasses in the processing plant. Sixty percent of sampled flocks at the age of slaughter were tested positive for *Campylobacter* at the farm. Results from processing plant showed that *Campylobacter*-negative flocks can get contaminated with *Campylobacter* strains from previously processed *Campylobacter*-positive flocks and that logistic scheduling may prevent cross-contamination. PFGE provided a useful tool to determine correlations between isolates from the farm and the isolates from the processing plants.

It has been reported that although various intervention strategies during processing have reduced *Campylobacter* contamination, they are still present on the carcass after processing at levels up to 2 to 4 $\log_{10}$ organisms/gm. These levels still pose threat to public health because of the low infective dose of *Campylobacter* as low as 500 organisms. However, our study showed that logistic slaughter has considerably reduced the numbers of *Campylobacter* on post-chill carcasses (0.2 to 1.5 $\log_{10}$ organisms/ml) and some flocks turned out to be negative. Through a combination of logistic slaughter and various reduction strategies public health risks of *Campylobacter* contamination could be considerably reduced from positively tested flocks. However, the effectiveness of this combined strategy relies on a highly sensitive and rapid test to determine colonization status of broiler flocks, and with a minimum delay between sampling and processing. Further research have to be carried out to achieve this goal.

REFERENCES

Aarts, H.J., L.A. Van Lith, and W.F. Jacobs-Reitsma, 1995. Discrepancy between Penner serotyping and polymerase chain reaction fingerprinting of *Campylobacter* isolated from poultry and other animal sources. ***Lett. Appl. Microbiol.*** 20(6):371–4.

Acuff, G.R., C. Vanderzant, M.O. Hanna, J.G. Ehlers, F.A. Golan, and F.A Gardner, 1986. Prevalence of *Campylobacter jejuni* in turkey carcass processing and further processing of turkey products. ***J. Food. Prot***. 49:712–7.

Aho, M., L. Nuotio, E. Nurmi, and T. Kliskinen, 1992. Competitive exclusion of campylobacters from poultry with K-bacteria and Broilact®. ***Int. J. Food Microbiol***. 15:265-275.

Aho, M., L. Nuotio, E. Nurmj, and T. Kliskinen, 1991. Development of a competitive exclusion flora active against campylobacters in poultry, ***Microb. Ecol. Health Dis***. 4:S79.

Allos, B.M., 2001. *Campylobacter jejuni* infections: update on emerging issues and trends. ***Clin. Infec. Dis***. 32:1201-6

Altekruse, S.F., N.J. Stern, P.I. Fields, and D.L. Swerdlow, 1999. *Campylobacter jejuni*-an emerging foodborne pathogen. ***Emerg. Infec. Dis***. 5:28-35

Bailey, J.S., J.E.Thomson, N.A.Cox, and A.D. Shackelford, 1986. Chlorine spray washing to reduce bacterial contamination of poultry processing equipment. ***Poultry Sci.*** 65:1120-3.

Bashor, M., K M Keener, P.A. Curtis, B.W. Sheldon, S. Kathariou, and J. Osborne, 2004. Effects of carcass washers on *Campylobacter* contamination in large broiler processing plants. ***Poultry Sci.*** 83(7):1232-9

Bashor, M., 2002. Effects of carcass washing systems on *Campylobacter* contamination in large broiler processing plants (MS, thesis). Raleigh, N.C: North Carolina State University. p 82.

Beery, J.T., M.B. Hugdahl, and M.P. Doyle, 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl. Environ. Microbiol*. 54(10): 2365–70.

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

Berndtson, E., M., Tivemo, and A. Engvall, 1992. Distribution and numbers of *Campylobacter* in newly slaughtered broiler chickens and hens. *Int. J. Food Microbiol.* 15:45-50

Berrang, M.E., and J.A. Dickens, 2000. Presence and level of *Campylobacter spp*. on broiler carcasses throughout the processing plant. *J. Appl. Poultry. Res*. 9:43–7.

Black, R.E., M.M. Levine, M.L. Clements, T.P. Hughes, and M.J. Blaser, 1988. Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis.* 157:472-479

Blaser M.J., 1995. *Campylobacter* and related species, p 1948-1956. *In* G.L. Mandell, J.E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, Inc., New York, N.Y.

Blaser, M.J., P.F. Smith, W.L.L.Wang, and J.C. Hoff, 1986. Inactivation of *Campylobacter jejun*i by chlorine and monochloramine. *Appl. Environ. Microbiol*. 51: 307-11.

Blaser, M.J., H.L. Hardesty, B. Powers, and W.L. Wang, 1980a. Survival of *Campylobacter fetus* subsp. *jejuni* in biological milieus. *J. Clin. Microbiol*. 11:309-313

Blaser, M.J., I.D. Berkowicz, F.M. Laforce, J. Cravens, L.B. Reller, and W.L. Wang, 1979. *Campylobacter* enteritis: clinical and epidemiological features. *Ann. Intern. Med.* 91:179-185.

Bolder, N. M., 1998. The microbiology of the slaughter and processing of poultry. p 159–173 .In The Microbiology of Meat and Poultry. A. Davies and R. Board, (ed.), Blackie Academic & Professional, London.

Bolton, F.J., D.N. Hutchinson, and D. Coates, 1984b. A. blood-free selective medium for the isolation of *Campylobacter jejuni* from feces. *J. Clin. Microbiol*. 19:169-171.

Bolton, F.J., D. Coates, P.M. Hinchcliffe, and L. Robertson, 1983. Comparison of selective media for isolation of *Campylobacter jejuni / coli*. *J. Clin. Pathol*. 36:78-83.

Bolton, F.J., and L. Robertson, 1982. A selective medium for isolating *Campylobacter jejuni / coli*. *J. Clin. Pathol*. 35:462-467.

Bryan, F.L. and P. Doyle, 1995. Health risks and consequences of *Salmonella* and *Campylobacter jejuni* in raw poultry. *J. Food Prot*.58:326-344

Butzler, J.P., M. De Boeck, and H. Goossens, 1983. New selective medium for isolation of *Campylobacter jejuni* from fecal specimens. Lancet i. 818.

Butzler, J.P., and M.B. Skirrow, 1979. *Campylobacter* enteritis. *Clin. Gastoneterol*. 8:737-765

Byrd, J.A., D.E Corrier, M.E. Hume, R.H. Bailey, L.H. Stanker, B.M. Hargis, 1998a. Incidence of *Campylobacter* in crops of preharvest market-age broiler chickens. *Poultry Sci.* 77:1303–5.

Corry J.E.L., D.E. Post, P. Colin, and M.J. Laisney, 1995. Culture media for the isolation of campylobacters. *Int. J. Food Microbiol*. 26:43-76

Evans, S.J., A.R. Sayers, 2000. A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. *Prev. Vet. Med.* 46(3):209–23.

Fernandez, H., M. Vergara, and F. Tapia. 1985. Dessication resistance in thermotolerant *Campylobacter* species. *Infection.* 13:197

Friedman, C.R., J. Neimann, H.C. Wegener, and R.V. Tauxe, 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. *In* Nachamkin, I., and M.J. Blaser, (ed.), *Campylobacter*. 2nd ed. Washington, D.C.: American Society for Microbiology Press. p121-38.

van de Giessen, A.W., J.J. Tilburg, W.S. Ritmeester,. and J. van der Plas, 1998. Reduction of *Campylobacter* infections in broiler flocks by application of hygiene measures. *Epidemiol. Infec.* 121:57-66.

George, H.A., P.S. Hoffman, R.M. Smibert, and N.R. Krieg, 1978. Improved media for growth and aerotolerance of *Campylobacter fetus*. *J. Clin. Microbiol*. 8:36-41.

Goossens, H., M. De Boeck, and J.P. Butzler, 1983. A new selective medium for the isolations of *Campylobacter jejuni* from human feces. *Eur. J. Clin. Microbiol*. 2:389-395.

Gregory, E., H. Barnhart, D.W. Dreesen, N.J. Stern, and J.L. Corn, 1997. Epidemiological study of *Campylobacter* spp. in broilers: Source, time of colonization and prevalence. *Avian Dis.* 41:890–8.

Harris, N.V., N.S. Weiss, and C.M. Nolan, 1986b. The role of poultry and meats in the etiology of *Campylobacter jejuni/coli* enteritis. *Am. J. Publ. Health* 76:407-411

Heuer, O.E., K. Pedersen, J.S. Andersen, and M. Madsen, 2001. Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. *Lett. Appl. Microbiol*. 33:269-274.

Hoffman, P., N.R. Krieg, and R.M. Smibert, 1979a. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. Jejuni. Physiological aspects of enhanced aerotolerance. *Can. J. Microbiol*. 25:1-7.

Hutchinson, D.N., and F.J. Bolton, 1984. Improved blood-free selective medium for the isolation of *Campylobacter jejuni* from fecal specimens. *J. Clin. Pathol*. 37:956-957.

Izat, A.L., F.A Gardner, J.H. Denton, and F.A. Golan, 1988. Incidence and level of *Campylobacter jejuni* in broiler processing. *Poultry. Sci*. 67:1568–72.

Jacobs-Rietsma, W., 2000. *Campylobacter* in the food supply. *In* Nachamkin, I and M.J. Blaster, (ed.) *Campylobacter*. 2nd ed. Washington, D.C.: ASM Press

Jacobs-Reitsma, W. F., 1997. Aspects of epidemiology of *Campylobacter* in poultry. *Vet. Quart.* 19:113–7.

Jacobs-Reitsma, W.F., 1995. *Campylobacter* bacteria in breeder flocks. *Avian Dis.* 39:355–9.

Juven, B.J., and I. Rosenthal, 1985. Effect of free-radical and oxygen scavengers on photochemically generated oxygen toxicity and on the aerotolerance of *Campylobacter jejuni. J. Appl. Bacteriol*. 59:413-419.

Karmali, M.A., A.E. Simor, M. Roscoe, P.C. Fleming, S.S. Smith, and J. Lane, 1986. Evaluation of blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from feces. *J. Clin. Microbiol*. 23:456-459.

Kemp, G. K., and K. R. Schneider. 2002. Reduction of *Campylobacter* contamination on broiler carcasses using acidified sodium chlorite. *Dairy Food Environ. Sanit.* 22:599–606.

Kemp, G.K, M.L. Aldrich, M.L. Guerra, and K.R. Schneider, 2001a. Continuous online processing of fecal- and ingesta-contaminated poultry carcasses using acidified sodium chlorite antimicrobial intervention. *J. Food Prot.* 64:807-16.

Lillard, H. S., 1982. Improved chilling systems for poultry. *Food Technol*. 36:58–67.

Line, J.E., 2001. *Campylobacter* and *Salmonella* levels for poultry raised on litter. J. *Poultry. Sci* 81(10):1473–81.

Line, J.E., 1999. Development of a selective differential agar for isolation and enumeration of *Campylobacter* spp. 10th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms. Baltimore MD.

Line, J.E., and M.E. Berrang, 2005. Comparison of two types of plating media for detection and enumeration of *Campylobacter* from poultry samples. *Int. J. Poul. Sci.* 4 (2): 81-84.

Mead, G.C., W.R. Hudson and M.H. Hinton, 1995. Effect of changes in processing to improve hygiene control on contamination of poultry carcasses with *Campylobacter*. *Epidemiol. Infec.*. 115: 495-500

Merino, F.J., A. Agulla, P.A. Villasante, A. Diaz, J.V. Saz, and A.C. Velasco, 1986. Comparative efficacy of seven selective media for isolating *Campylobacter jejuni*. *J. Clin. Microbiol*. 24:451-452.

Miwa, N., Y. Takegahara, K. Terai, H. Kato, and T. Takeuchi, 2003. *Campylobacter jejuni* contamination on broiler carcasses of *C. jejuni*-negative flocks during processing in a Japanese slaughterhouse. *Int. J. Food Microbiol.* 84:105-109.

Nachamkin, I., B.M. Allos, and T.W. Ho, 2000. *Campylobacter jejuni* infection and the association with Guillain-Barre Syndrome, p. 155-175. *In* Nachamkin. I and M.J. Blaser (ed), *Campylobacter*. ASM Press, Washington, D.C.

Nachamkin, I., 1995. *Campylobacter* and *Arcobacter.* p.483-491. *In* Manual of Clinical Microbiology, 6th ed. P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, and R.H. Yolken, (ed.) American Society for Microbiology, Washington, D.C.

Newell, D. G., and C. Fearnley. 2003. Source of *Campylobacter* colonization in broiler chickens. *Appl. Environ. Microbiol*. 69:4343-4351.

Newell, D.G., J.E. Shreeve, M. Toszhegy, G. Domingue, S. Bull, T. Humphrey, and G. Mead, 2001. Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. *Appl. Environ. Microbiol*. 67:2636-2640

Newell, D.G., J.A. Wagenaar, 2000. Poultry infections and their control at the farm level. *In*: Nachamkin, I., M.J. Blaser, (ed.) *Campylobacter.* 2nd ed. Washington, D.C.: ASM Press. P 497–510.

Oyarzabal, O.A., K S. Macklin, J.M. Barbaree, and R.S. Miller, 2005. Evaluation of agar plates for direct enumeration of *Campylobacter* spp. from poultry carcass rinses. *Appl. Environ. Microbiol.* 71(6):3351–3354.

Padungtod, P., R. Hanson, D.L. Wilson, J. Bell, J.E. Linz and J.B. Kaneene, 2002. Identification of *Campylobacter jejuni* isolates from cloacal and carcass swabs of chickens in Thailand by a 5'nuclease fluorogenic polymerase chain reaction assay. *J. Food Prot.* 65:1712-1716

Park, S.F., 2002. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int. J. Food Microbiol*. 74:177-188

Pattison, M**.,** 2001. Practical intervention strategies for *Campylobacter*. *Symp. Ser. Soc. Appl. Microbiol.* 30:121S–125S.

Rivoal, K., M. Denis, G. Salvat, P. Colin, G. Erme, 1999. Molecular characterization of the diversity of *Campylobacter* spp. isolates collected from a poultry slaughterhouse: analysis of cross-contamination. *Lett. Appl. Microbiol*. 29:370-374

Robinson, D.A., 1981. Infective dose of *Campylobacter jejuni* in milk. *Br. Med. J.* 282:1584.

Schoeni, J. L., and M. P. Doyle, 1992. Reduction of *Campylobacter jejuni* colonizations of chicks by cecum-colonizing bacteria producing anti-*C. jejuni* metabolites. *Appl. Environ. Microbiol.* 59:664-670.

Shallow, S., P. Daily, G. Rothrock, A. Reingold, D. Vugia, S. Waterman, T. Florentino, R. Marcus, R. Ryder, P. Mshar, J.L. Hadler, W. Vaughman, J. Hogan, V. Deneen, C. Hedberg, M.T. Osterholm, M. Cassidy, J. Towens, B. Shiferaw, P. Cieslak, K. Hedberg, and D. Fleming, 1997. Foodborne diseases active surveillance network, 1996. *Morb. Mortal. Weekly Rep*. 46:258-261

Shanker, S., A. Lee, and T. C. Sorrell, 1990. Horizontal transmissions of *Campylobacter jejuni* amongst broiler chicks: experimental studies. *Epidemiol. Infec*. 104:101-110.

Skirrow, M.B., J. Benjamin, M.H. Razi, and S. Waterman, 1982. Isolation, cultivation and identification of *Campylobacter jejuni* and *Campylobacter coli*. *In*: Corry, J.E.L., D. Roberts, and F.A. Skinner, (ed.) Isolation and Identification Methods for Food Poisoning Organisms. Academic Press, London, p 313-328.

Skirrow, M.B., 1977. *Campylobacter* enteritis: a new disease. *Brit. Med. J.* 2:9-11.

Slader, J., G. Domingue, F. Jorgensen, K. McAlpine, R. J. Owen, F. J. Bolton, and T. J. Humphrey, 2002. Impact of transport crate reuse and of catching and processing on

Campylobacter and *Salmonella* contamination of broiler chickens. *Appl. Environ. Microbiol.* 68:713-719.

Slavik, M.F., J.W. Kim, M.D. Pharr, D.P. Raben, S. Tsai, and C.M. Lobsinger, 1994. Effect of trisodium phosphate on *Campylobacter* attached to post-chill chicken carcasses. *J. Food. Prot*. 57:324–6.

Smith, T., and M.S. Taylor, 1919. Some morphological and biochemical characters of the spirilla (Vibrio fetus n. sp.) associated with disease of the fetal membranes in cattle. *J. Exp. Med.* 30:299-312

Stern, N.J., 1992. Reservoirs for *Campylobacter jejuni* and approaches for intervention in poultry. *In*: Nachamkin, I., M.J. Blaser, L.S. Tomkins, (ed.) *Campylobacter jejuni*: current status and future trends. Washington, D.C.: ASM Press. p 49–60.

Stern, N.J., P.J. Fedorka-Cray, J.S. Bailey, N.A. Cox, S.E. Craven, K.L. Hiett, M.T. Musgrove, S. Ladley, D. Cosby, and G.C. Mead, 2001. Distribution of *Campylobacter* spp. in selected U.S. poultry production and processing operations. *J. Food Prot.* 64(11): 1705-10.

Stern, N.J., M.R.S. Clavero, J.S. Bailey, N.A Cox and M.C. Robach, 1995. *Campylobacter* spp. in broilers on the farm and after transport. *Poultry Sci.* 74: 937-941

Stern, N.J., C.E. Lyons, and M.T. Musgrove, 1995b. Bacterial quality of broilers and alternative processing procedures. *J. Appl. Poultry. Res* 4:164–9.

Stern, N. J., B. Wojton, and K. Kwiatek**,** 1992. A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni. J. Food Prot.* 55**:**515-517.

Stern, N. J., J. Stan Bailey, L. C. Blankenship, N. A. Cox, and F. McHan, 1988. Colonization characteristics of *Campylobacter jejuni* in chick ceca. *Avian Dis.* 32:330-334.

Tauxe, R.V., 1992. Epidemiology of *Campylobacter jejuni* infections in the United States and other nations. *In*: Nachamkin, I., M.J. Blaser, L.S. Tomkins, (ed.) *Campylobacter jejuni*: current status and future trends. Washington, DC: American Society for Microbiology. p 9–19.

U.S. Dept. of Agriculture (USDA), 1995. Pathogen reduction; hazard analysis and critical control point (HACCP) systems. FSIS proposed rule, FSIS final rule. 60 FR 6774,6794-6795. Washington, D.C.: U.S. Government Printing Office.

Weinrich, K., K. Winkler, and E. Heberer, 1990. Untersuchungen zur Eignung ausgewahlter Huminsaurenchargen asl Nahbodenzusatz fur Medien sur Isolierung thermophiler *Campylobacter* species. *Deusche Tierarztliche Wochenschrift* 97:511-515.

Wesley, R.D., B. Swaminathan, and W.J. Stadelman, 1983. Isolation and enumeration of *Campylobacter jejuni* from poultry products by a selective enrichment method. *Appl. Environ. Microbiol.* 46:1097-1102.

Waldroup, A.L., B.M. Rathgeber, R.E. Hierholzer, L. Smoot, L.M. Martin, D.L. Bilgili, D.L. Fletcher, T.C.Chen, and C.J. Wabeck, 1993. Effects of reprocessing on microbiological quality of commercial pre-chill broiler carcasses. *J. Appl. Poultry. Res.* 2(2):111–6.

Waldroup, A.L., B.M. Rathgeber and R.H. Forsythe, 1992. Effects of six modifications on the incidence and levels of spoilage and pathogenic microorganisms on commercially processed post-chill broilers. *J. Appl. Poul. Res.*1:226-234

Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. G. 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**:**5431-5436

Zutter, L.D., L. Herman, and M. Hendrickx, 2005. Logistic slaughter still allows carcass contamination. *In* World Poultry: *Salmonella* and *Campylobacter* special.