

DIRECT MICROSCOPIC OBSERVATION AND VISUALIZATION  
OF *CAMPYLOBACTER JEJUNI* VIABILITY ON CHICKEN SKIN

by

WALAIRUT CHANTARAPANONT

(Under the Direction of JOSEPH F. FRANK)

ABSTRACT

A method to detect viability of *Campylobacter jejuni* on chicken skin was developed by employing confocal laser scanning laser microscopy (CSLM) visualization of *Campylobacter jejuni* transformed with P<sub>cgfp</sub> plasmid (GFP-*Campylobacter*) exposed to 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a redox dye, which is taken up, reduced to CTC-formazan, and accumulated intracellularly in respiratory active cells. The data indicated that GFP-*Campylobacter* remaining on the chicken skin surface after rinsing were mostly located in crevices, entrapped inside feather follicles with water and also entrapped in the surface water layer. Most of the viable cells were entrapped with water in the skin crevices and feather follicles. The population of *C. jejuni* decreased during storage at 25°C for 24hr. There was no effect of 25°C storage on viability of *C. jejuni* located 20-30µm beneath the chicken skin. The log<sub>10</sub> population of *C. jejuni* on chicken skin stored at 4°C slightly increased from 0hr to 72hr. The viable cells of GFP-*C. jejuni* located at the surface and 20-30µm beneath the chicken skin increased. Live and dead campylobacters are initially retained with water on the skin and penetrate into the skin with time. Chicken skin provides a suitable environment for campylobacter to survive and grow at 25°C and 4°C, respectively. The effectiveness of chlorine, acidified sodium chlorite and peracetic acid on *C. jejuni* viability on chicken skin were determined. All sanitizers were equally effective in reducing *C. jejuni* on chicken skin. The effectiveness of these sanitizers in killing *C. jejuni* on chicken skin at different locations could not be differentiated in statistical analysis. The detection of live cells after treatment by direct microscopic count at deeper locations has a limitation because of the low number of cells. However, elimination *C. jejuni* from chicken skin was not achieved as shown by direct microscopic count and plate count.

INDEX WORDS: *Campylobacter jejuni*, confocal scanning laser microscope, CSLM, chicken skin, chlorine, peracetic acid, acidified sodium chlorite, CTC

DIRECT MICROSCOPIC OBSERVATION AND VISUALIZATION  
OF *CAMPYLOBACTER JEJUNI* VIABILITY ON CHICKEN SKIN

by

WALAIRUT CHANTARAPANONT

B.Sc., Kasetsart University, Thailand, 1995

M.S., The University of Georgia, 1999

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial  
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2002

© 2002

Walairut Chantarapanont

All Rights Reserved

DIRECT MICROSCOPIC OBSERVATION AND VISUALIZATION  
OF *CAMPYLOBACTER JEJUNI* VIABILITY ON CHICKEN SKIN

by

WALAIRUT CHANTARAPANONT

Major Professor: Joseph F. Frank

Committee: Larry R. Beuchat  
Mark A. Farmer  
Ronald R. Eitenmiller  
William L. Kerr

Electronic Version Approved:

Maureen Grasso  
Dean of the Graduate School  
The University of Georgia  
December 2002

## ACKNOWLEDGEMENTS

I would like to thank to Dr. Joseph F. Frank, my major professor, for all his guidance in my research and help me complete my dissertation, Dr. Mark Berrang for his support in my research, Dr. Larry R. Beuchat, Dr. Mark A. Farmer, Dr. Ronald R. Eitenmiller and Dr. William L. Kerr, who are served on my Advisory Committee, Dr. Rick Meinersmann for providing GFP-*Campylobacter jejuni* and laboratory space for my study. Thank you to my father and mother who always love and support me to work towards my degree, also Voravut Boonyoungyein for all his support, and Parthana Parthanadee, Pensiri Pankam, Puntarika Rattanatriwong, Uruchaya Sonchaeng, Varapha Lotong, Marisa Chamruspollert, and Dayin Koompalum for being my best friends.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS .....	iv
INTRODUCTION .....	1
LITERATURE REVIEW .....	3
CHAPTER	
1    DIRECT MICROSCOPIC OBSERVATION OF <i>CAMPYLOBACTER JEJUNI</i> VIABILITY ON CHICKEN SKIN.....	52
2    DIRECT MICROSCOPIC OBSERVATION OF <i>CAMPYLOBACTER JEJUNI</i> SURVIVAL ON CHICKEN SKIN AS AFFECTED BY TEMPERATURE .....	73
3    DIRECT MICROSCOPIC OBSERVATION IN EFFICACY OF SELECTED SANITIZERS FOR INACTIVATION OF <i>CAMPYLOBACTER JEJUNI</i> ON CHICKEN SKIN .....	90
CONCLUSION .....	118

## INTRODUCTION

Clinical and epidemiologic investigations have established *C. jejuni* as one of the most common causes of sporadic bacterial enteritis in the United States. Over 2 million cases are estimated to occur annually, an incidence of infection similar to that in the United Kingdom and other developed nations. Mortality associated with *Campylobacter* infections in the United States is estimated of the U.S. Centers for Disease Control to be between 200 and 730 deaths per year (Tauxe, 1992). Pathogenesis of *C. jejuni* depends on pathogen-specific and host related factors including age, health, and immune response. Due to these factors, the lowest infective dose for *Campylobacter* has not established; however, 500 to 800 organisms were sufficient to make healthy young adults ill in a volunteer study (Black et al., 1998 and Robertson, 1981).

Epidemiology (case-control) studies show a significant association between *Campylobacter* infection in humans with handling and eating raw or undercooked poultry. Large percentages of fresh and even frozen poultry products are contaminated with *Campylobacter*. Levels of contamination may vary between log<sub>2</sub> and log<sub>5</sub> cfu per carcass or log<sub>10</sub>1 to log<sub>6</sub> cfu/ 100g of meat, depending on the study and the analytical method. However, with the relatively low infectious dose of 500-800 cfu reported for *Campylobacter*, poultry products may pose a high risk for consumers if handled unhygienically during preparation or insufficiently cooked. *Campylobacter jejuni* are

generally commensal in the intestinal tract of a wide variety of animals especially poultry. The chicken intestinal tract, ceca and colon, which contain high levels of *C. jejuni* may leak during processing, especially during defeathering and evisceration which result in adding *C. jejuni* contamination to the skin of the carcass and remain on the skin throughout the poultry processing.

In order to understand the role of chicken skin in protecting *C. jejuni*, the main objective of this study was to develop a method to detect viability of *C. jejuni* at different locations on chicken skin. This method employs confocal laser scanning laser microscopy (CSLM) visualization of *Campylobacter jejuni* transformed with P<sub>cgfp</sub> plasmid (GFP-*Campylobacter*) exposed to 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a redox dye which is taken up, reduced to CTC-formazan, and accumulated intracellularly in respiratory active cells. This dissertation consists of three chapters. The objective of the first chapter is to identify the specific sites and locations on chicken skin, which provide the suitable environment for *C. jejuni* to survive. The objective of the second chapter is to detect the survival of GFP-*Campylobacter jejuni* cells at different depths on chicken skin stored at different temperatures. The information from chapter 1 and chapter 2 will help explain how *C. jejuni* survives on chicken skin, and may be useful in the development of technologies for eliminating *C. jejuni* from the skin. And the objective of the last chapter is to determine the efficacy of selected sanitizers in inactivation *C. jejuni* on chicken skin at different depths.

#### REFERENCES:

- Black, R. E., M. M. Levine, M. L. Clements, P. Timothy. 1998. Experimental *Campylobacter jejuni* infections in humans. J. Infect. Dis. 157:472-479.
- Robertson, D. A. 1981. Infective dose of *Campylobacter jejuni* in milk. Br. Med. J. 1:1594.



## LITERATURE REVIEW

### ***Campylobacter* spp.**

#### **General characteristics**

The genus *Campylobacter* was first isolated and classified as *Vibrio fetus* because of its morphology. Later, it was found to be unrelated to the family Vibrionaceae on the basis of its different nucleotide base composition, as well as its inability to use sugars either oxidatively or fermentively. A new genus, *Campylobacter*, was created to exclude *V. fetus* from *Vibrio* species (26). The genus *Campylobacter* consists of about 20 species and subspecies (103). *C. jejuni* spp. *jejuni* referred to as *C. jejuni* is the species of greatest concern to the food microbiologist. *C. jejuni*, *C. coli*, and *C. lari* cause more than 90% of the reported human disease. The differentiating characteristics of *Campylobacter* species associated with human diseases are presented at Table 1. Sensitivity to nalidixic acid and ability to hydrolyse hippurate is the characteristic of *C. jejuni*, which distinguish *C. jejuni* from *C. laridis* and *C. coli*, respectively.

*Campylobacter* species are Gram-negative, have narrow (0.2-0.5 micron), long (1.5-5.0 micron) rods that are spiral-shaped and nonspore-forming, and contain a single polar flagellum on one or both ends that produces the corkscrew-like motility. Motility is best observed with phase contrast or darkfield microscopy (90). These species grow well at temperatures ranging from 37°C to 42°C, but should not be considered thermophilic

because they die at temperatures at or above 45°C. Older cultures often become coccoidal. It is unclear whether this morphological change confers an advantage toward survival or whether this is cellular deterioration leading eventually to death. Viable, nonculturable forms may arise under conditions of starvation.

Table 1: Differentiating Characteristics of *Campylobacter* Species (52)

	<i>C.jejuni</i>	<i>C. coli</i>	<i>C. laridis</i>	<i>C. fetus</i> subsp. <i>fetus</i>
Growth				
25°C	-	-	-	+
37°C	+	+	+	+
42°C	+	+	+	-
Catalase	+	+	+	+
Nitrate Reduction	+	+	+	+
H <sub>2</sub> S (TSI Agar)	-	-	-	-
Hippurate Hydrolysis	+	-	-	-
Urease	-	-	-	-
Susceptibility				
Nalidixic Acid	S	S	R	R
Cephalonin	R	R	R	S

+ = Positive reaction, - = Negative reaction, R = Resistant, S = Susceptible.

*Campylobacter*s are oxidase-positive and reduce nitrate to nitrite. Their energy is derived through accessing a variety of tricarboxylic acid (TCA) intermediates or by decarboxylation and deamination of amino acids, not through carbohydrate utilization. Although oxygen is characteristically the terminal electron acceptor in the TCA cycle, the organisms do not tolerate levels of oxygen normally found in the atmosphere, but grow optimally in microaerobic conditions. They require a low oxygen tension (3% to 15%) and an increased CO<sub>2</sub> level (3% to 5%) for growth. They are extremely sensitive to hydrogen peroxide and superoxide ions as little as 0.00124% hydrogen peroxide that appear in the culture medium when it is exposed to air and light. However, addition of

0.025% each of ferrous sulfate, sodium metabisulfate and sodium pyruvate (FBP) to culture media will increase the aerotolerance of the culture by destroying hydrogen peroxide and superoxide anions that appear in the medium when it is exposed to air and light (45, 55, 56). Addition of blood, which contains catalase and superoxide dismutase, or bovine superoxide dismutase and catalase to culture media also enhance oxygen tolerance of campylobacters (55). Most enteropathogenic campylobacters are catalase-positive. The organisms are thus well suited to their primary niches in the reproductive and intestinal tracts of warm-blooded animals.

#### **Incidence of *Campylobacter* spp.:**

*Campylobacter* spp. generally are commensal in the intestinal tract of a wide variety of animals; however, they occasionally serve as enteric pathogens in the young of some species such as calves, lambs, and puppies. Infections caused by these organisms are of primary interest because of the serious economic losses to farmers that results from abortions and infertility of infected cattle and sheep, but they are now recognized as a cause of human illness. *C. jejuni* has the broadest animal reservoir, which includes poultry, cattle, sheep, swine, fowl, dogs, cats and monkeys; *C. coli* is found primarily in swine; *C. fetus* is isolated from sheep and cattle, and *C. lariidis* from seagulls (68). In addition, campylobacters can be isolated from seawater, streams, and rivers that have been tainted by fecal contamination from wild and domestic animals.

**Epidemiology:**

Both *Campylobacter fetus* subsp. *fetus* and *Campylobacter jejuni* were isolated from blood cultures of human in 1957 but were thought to be rare and perhaps opportunistic pathogens (76). The development of selective *Campylobacter* stool culture media by Skirrow and by Blaser led to the recognition that *Campylobacter* was a common cause of human diarrhea (14, 126). After that this pathogen was identified as the most frequent cause of acute bacterial diarrhea in many countries (16). Many clinical and epidemiologic investigation have established *C. jejuni* as one of the most common causes of sporadic bacterial enteritis in the United States. Over 2 million cases are estimated to occur annually, an incidence of infection similar to that in the United Kingdom and other developed nations (137). Mortality associated with *Campylobacter* infections in the United States is estimated of the U.S. Centers for Disease Control to be between 200 and 730 deaths per year by Tauxe (1992). The highest incidence is in infants and young children and a second peak in adults 20 to 40 years old. In the developing countries, the incidence is not known, but based on high isolation rates, it is likely to be 5-10 times higher than in developed countries (46, 48, 138).

Most of the *Campylobacter* infections are food- or water borne. In the past, most outbreaks of campylobacteriosis often associated with the consumption of raw, unpasteurized milk or milk that has been contaminated postpasteurization and insufficiently cooked poultry also has been implicated (43). There is increasing evidence worldwide showing an association between contamination of poultry and human illness. *C. jejuni* serogroups isolated from poultry sources are the same as those most frequently

isolated from human patients whereas campylobacter isolates from swine (*C. coli*) do not belong to serotype found in humans (115, 118).

**Pathogenesis:**

Pathogenesis of *C. jejuni* depends on pathogen-specific and host related factors including age, health, and immune response. Due to these factors, the lowest infective dose for *Campylobacter* has not established; however, 500 to 800 organisms were sufficient to make healthy young adults ill in a volunteer study (13, 116). Human infection with *C. jejuni* can result in a range of clinical illness from transient asymptomatic colonization to severe dysentery. Stools are commonly watery and, after 1 to 2 days of diarrhea, often contain fresh blood (23). Diarrhea is the most common symptom and is usually accompanied by malaise, fever, abdominal pain, nausea and vomiting, bloody diarrhea and joint pain. Onset of diarrhea generally occurs 2 to 5 days after oral exposure and generally lasts 5 to 10 days, and resolution of other signs usually occurs by 2 weeks after onset (139). Chronic illness can occur within 1-2 weeks in approximately 1% of patients appearing in the form of reactive arthritis (4, 127). An estimated one in every 1,000 patients of Campylobacteriosis contract Guillain-Baire syndrome (peripheral polyneuropathy), a more serious side effect, which is characterized by rapid progressive symmetric paralysis (4, 67, 153).

**Mechanism of Pathogenesis:**

The mechanism of pathogenesis is unclear. Clinical symptoms of *Campylobacter* infections resemble those manifested by other enteropathogens, so it is reasonable to

presume that similar mechanisms are involved. *Campylobacter* infection of humans results in high titers of humoral IgG, IgM, and IgA. This indicates that bacterial cellular constituents pass from the intestinal tract across the epithelial. The mechanisms of pathogenesis of *Campylobacter* may include association with and attachment to the intestinal epithelium, invasion of the epithelium, and/or toxin formation (79). Adherence is related to an outer membrane protein (OMP) with a molecular weight of 27 kDa that binds to the host membrane. The outer membrane protein may be a major virulence factor. *Campylobacter* flagella served a critical role in the colonization (104). Mutants without flagella were unable to colonize, while the wild type readily did. Szymanski et al (1995) also showed that motility played several roles in *C. jejuni* pathogenesis, which included tissue tropism, penetration of mucus lining the intestinal tract, and increasing the efficiency of *C. jejuni* attachment to host epithelial cells (134).

Strains of *Campylobacter* isolated from patients with watery diarrhea produce a heat-labile enterotoxin that is structurally and immunologically related to the cholera enterotoxin. It causes a secretory diarrhea by stimulating adenylase cyclase activity in the intestinal mucosa and disrupting the normal ion transport in the enterocytes. A cytotoxin also is produced by some isolates of *C. jejuni* and *C. coli*. It injures a variety of mammalian cells, but its role in human diarrheal disease is unknown (52). Elongation, shortening and blebbing of microvilli and extrusion of apical cytoplasm are likely due to cytotoxin produced by *C. jejuni* within the intestinal lumen (58).

### **Factors affecting *C. jejuni* growth or survival:**

Temperature, which includes heating, refrigeration, freezing, pH, salinity, preservatives, atmospheric condition, irradiation, disinfectants, and desiccation all affect the survival of *C. jejuni*. Growth of campylobacters in food is unlikely, though survival of the organism on contaminated foods is a concern in view of the low infective dose.

**- *Temperature:*** *Campylobacter* grows at temperatures between 30 and 45°C, with the optimum growth at 42 to 43°C. It dies more rapid at 25°C than at either 4°C or 30°C. Heat injury of *C. jejuni* occurs at 46°C and thermal inactivity occurs at 48°C. The decimal reduction time for campylobacters at 55°C is about 1 min and the z value is about 5°C in a skim milk heating media (38). Properly pasteurized milk, normally pasteurized at 62.7°C for 30 min or 71.7°C for 15 sec, should not contain viable cells of *C. jejuni*.

**- *pH:*** Optimal growth occurs at pH 6.5 to 7.5, with good growth at pH 5.5 to 8.0, and no growth below pH 5.1 (38, 47). At pH 3.0 to 4.5, the rate of cell death decreases with decrease in temperature (47).

**- *Salinity:*** Campylobacters are highly sensitive to NaCl (optimum growth concentration is 0.5%); even moderate levels of NaCl in foods can increase death of *C. jejuni*. Sensitivity to NaCl is also temperature dependent. The lethal action of NaCl, like that of other food preservatives, is known to be reduced at low temperature (60). However, *C. jejuni* tolerates more NaCl in the growth medium nearer the optimum growth temperature of 42°C than at 30°C (129).

**- *Spices:*** The presence of spices can affect survivability of *C. jejuni* (34). However, given the length of time required for substantial reductions in numbers to occur

and the level of spices tested, the practical application of the effect is still questionable. Some effect may occur on the surface of a spiced meat product, where exposure to drying and oxygen occur, but these conditions alone are lethal to campylobacters.

**- Irradiation:** *Campylobacter* is more sensitive to irradiation than either *Listeria monocytogenes* or *Salmonella*, so treatments sufficient to eliminate these pathogens from poultry carcasses would also eliminate campylobacters (112). Campylobacters could not survive a dose of 1 kGy in chicken paste (136). *C. jejuni* was more resistant at subfreezing temperatures in ground meats than at temperatures above freezing, which is probably due to a decrease in the diffusion of free radical from the immobilization of the water phase (2).

**- Disinfectant:** Most common disinfecting agents at standard concentrations can kill *Campylobacter* (149).

### ***Campylobacter* in Poultry Production and Processing:**

Epidemiology (case-control) studies show a significant association between *Campylobacter* infection in humans with handling and eating raw or undercooked poultry (37, 53). However, the extent to which poultry consumption is responsible for human campylobacteriosis is not exactly known (64). Many *Campylobacter* researches have been poultry related. Large percentages of fresh and even frozen poultry products are contaminated with *Campylobacter* (139). Levels of contamination may vary between  $\log_{10}2$  and  $\log_{10}5$  cfu per carcass or  $\log_{10}1$  to  $\log_{10}6$  cfu/ 100g of meat, depending on the study and the analytical method (10). A direct correlation of various studies may be difficult to make due to different sampling methods (64). However, with the relatively



low infectious dose of 500-800 cfu reported for *Campylobacter*, poultry products may pose a high risk for consumers if handled unhygienically during preparation or insufficiently cooked.

**1). *C. jejuni* & poultry on farms:**

*C. jejuni* associates with poultry, including broilers, laying hens, turkeys, and ducks, during their life cycle of laying, hatching, brooding and rearing. The colonization rate in poultry varies from 0 to 100 percent, while the median is 62% positive (1, 15, 44, 65, 151, 155). In addition, a large number of *C. jejuni* can colonize poultry without showing any signs of clinical illness. The presence of  $\geq 1,000,000$  *C. jejuni* per gram of feces is not unusual. Fecal material residues, dust, and fluff in the hatchery may be an additional source of pathogens that contaminate young poultry.

**- Intestinal tract:** After hatching, the intestinal tract of poultry becomes colonized by Enterobacteriaceae (including coliforms), clostridia and fecal streptococci within a day (51). Later, these organisms are replaced by a characteristic microflora in different regions of the intestines. *C. jejuni* can be present in the lower intestinal tract in populations as high as  $10^7$  per g. It colonizes primarily the lower gastrointestinal tract of chicks, principally the ceca, large intestine and cloaca where densely packed cells localized in mucus within crypts (8). Colonization levels in the small intestinal, especially the ceca, range from  $10^5$  to  $> 10^9$  cfu/g (10, 98). Campylobacters appear to pervade the lamina of crypts without attachment to crypt microvilli. *Campylobacter jejuni* is chemoattracted to mucin and L-fucose, an exposed carbohydrate constituent of mucin (57). The chemoattraction of campylobacters to mucin may attract the bacteria to

mucus, in which they can move by highly active flagella to mucus-filled crypts where the organisms establish themselves. Campylobacters likely remain in the crypts because of their attraction to mucin and *C. jejuni* can also utilize mucin as a sole substrate for growth.

- **Feed:** Feed is an unlikely source of *C. jejuni* because of its low moisture content will cause death of campylobacters.

- **Environment:** There are many possible ways that poultry can become contaminated with *C. jejuni* from environment. In addition, poultry have a pecking habit which pathogens shed in feces and present in litter and soil to be ingested. That makes them prone to become colonized. There is evidence that even though the feed, litter and water might be negative for *Campylobacter* prior to infection of live birds, contamination might spread from an infected flock of birds to succeeding flocks via litter (44). *C. jejuni* can survive in an aqueous environment where it can remain viable up to 3 to 4 weeks (17). Water contaminated with the pathogen might be a prime source of infection of chicks (114). Bacterial pathogens also can survive in water and may multiply if organic matter such as feed residues or fecal matters are present. Campylobacters have been isolated from water receptacles used by poultry (70, 123). Skin and feathers can readily become contaminated in the dust environment of the places poultry are kept during brooding and raising, and feet become contaminated from feces in litter and from water (61).

## **2). During transportation from farm to slaughter:**

Transmission of microorganisms between birds continues during the interval between leaving the farm and arrival at slaughtering facilities. Although feed is often withheld from poultry a few hours before transport to reduce the amount of fecal droppings, considerable defecation still occurs during transit. Stress caused by gathering, transporting, crowding and holding in crates before slaughter, and inclement weather increases contamination (96). Stern et. al. (1995) indicated that transport and holding prior to processing contributes to the *Campylobacter* spp. of  $10^4$  cfu higher than normally found on processed poultry carcasses with the chance of 12.1% of the individual birds contaminated pretransport to 56% of the individuals contaminated posttransport, which was greater than 100-fold (133).

## **3). During processing:**

Within processing plants, carcasses pass through a series of operations where contamination can occur from the environment and equipment of the plant, hands of workers and cross-contamination from other birds. The various processing steps either increase or decrease microbial contamination. Jones et al (1991) isolated *Campylobacter* from 20% of the cloacal swabs as birds entered the plant, 52% after immersion chilling and 31.6% at retail (65). Before scalding, the incidence of *E. coli*, *Salmonella* spp., and *C. jejuni/coli* on feathers, breast skin, and feet were higher than on thigh and on drum skin. In addition, the incidence of *C. jejuni* was higher on feathers and skin of breast than on other feather and skin locations (80). It showed that the organisms could be spread

either from within intestinal tract, or from the bacteria on the external surfaces of the bird occurring during processing.

**- Pre-slaughtering operations:** Populations of *C. jejuni* on skin surfaces of incoming chickens are approximately 1 to 5.5 cfu per cm<sup>2</sup> (63). Considerable amount of dust are generated and becomes airborne while birds struggle when grabbed and while being shackled (111). The dust settles on birds that pass through this environment. During electrical stunning, there is considerable defecation that soils the cloacal opening and nearby skin and feathers.

**- Scalding:** Scalding of the birds, necessary to facilitate defeathering, is usually employed at water temperature between 50 to 60°C applied several minutes. During scalding, some pathogens on feathers, feet and skin are washed into the scald water where they are either (a) killed by the high temperature of the water; (b) float away in overflow water; or (c) survive and redistribute on the same or other carcasses (21). Water temperature greatly affects die-off rate and influences survivor of both *Salmonella* and *C. jejuni*. Greater reductions occur in scald baths with water temperatures of 58 to 60°C than at 52°C (61, 100, 105, 106, 108). At higher temperatures (hard scalds e.g. 58°C for about 2.5 minutes), the entire epidermis is removed by the plucking process, and the exposed dermal tissue provides not only a new surface for colonization of microorganisms in the way that contaminating bacteria attach more firmly to the skin, but also a more suitable substrate for the growth of spoilage microorganisms (144). In comparison, the epidermis may be damaged but is not removed during scalding at lower temperature (soft scalds e.g. 52°C for about 3 minutes) and subsequent plucking. However, during scalding, soil, dust, and fecal matter from feet, feathers, skin, intestinal

tract and respiratory tract are released into the scald water. Spoilage as well as pathogenic microorganisms have been isolated from scald tank water, from carcasses and air sacs immediately after scalding. Time and temperature of scalding can influence the extent of microbial destruction and types of microbes that survive on carcasses. High temperature, 60°C for 115s, can significantly decrease number of Enterobacteriaceae, psychrotrophic microorganisms, *E. coli*, and total aerobic plate count (97). Scalding is neither a site of significant contamination nor a step that can be relied upon to reduce the prevalence of *C. jejuni* on poultry carcasses (44, 109, 151, 155).

**- Defeathering (picking or plucking):** Considerable contamination of poultry skin by salmonellae and campylobacters occurs during defeathering (61, 63, 94, 96, 108, 151, 155). This is a major point (hazard) of transfer of soil- and fecal-borne pathogens, as well as those that reside on feathers and skin. During defeathering and evisceration, leakage of intestinal contents almost inevitably contributes to the contamination of carcasses. Berrang et. al. (2001) showed that increasing recovery of *Campylobacter* after defeathering was due to the escape of contamination from the cloaca (11). Pathogen populations on the finished packaged product often reflect the contamination present immediately after picking. Microorganisms from one bird can contaminate many birds that follow, and microorganisms from them contaminate others. This cross-contamination involving many carcasses has been demonstrated with indicator bacteria (100). The continuous beating of the contaminated rubber fingers on skin surfaces push microorganisms into skin tissues, crevices and feather follicles. Then, they resist removal during subsequent washing operations. Moreover, aerosols are generated during this operation, and microorganisms within them reach carcasses, workers and other

environmental surfaces as the aerosols settle (111). Defeathering equipment is difficult to clean and is not cleaned until the end of day's operation, and often the cleaning does not remove all microbial contaminants and organic matter. As time passes, surviving bacteria can multiply in the moist, soiled, and cracked surfaces, and then survive on the dried surface for several days.

- **Evisceration:** During evisceration, transfer of microorganisms continues from carcasses to hands of workers and equipment and utensil surfaces that the carcasses contact, and from them to other carcasses. Cutting of the intestinal tract or rough handling during pulling viscera by worker as well as mechanical equipment also spreads fecal contamination (6, 99). Vacuuming the vent and cavity removes some faecal and other contamination. Proper maintenance and continuous cleaning of machinery are needed to prevent an increase in microbial population on poultry carcasses (125).

- **Washing:** Spray washing carcasses removes loose organic matter and with it some pathogens, and replaces the heavily contaminated surface fluid with cleaner water. The reduction of bacteria is usually limited to 90% (96). The remaining bacteria are either entrapped within or adhere to skin or flesh surfaces. Spraying carcasses several times during evisceration is more effective for preventing an increase in Enterobacteriaceae and salmonellae than a single wash after evisceration (101). Adding organic acids or chlorine to these sprays does not extend the shelf-life of fresh poultry but when chlorine is added to both the spray water and chill tank water can lower bacterial numbers (66).

- **Chilling:** The purpose of chilling is to delay growth of psychrotrophic spoilage bacteria and prevent growth of most foodborne pathogens. Poultry carcasses can be

chilled by immersion in tanks of cold water with or without the addition of ice, by sprays of cold water or by circulation of cold air. The method selected is influenced by the relative impact of economics, hygiene and local regulations. The ideal method will provide chilling at the least cost without causing microbial contamination or permitting multiplication. Chilling does not generally involve decontamination to further improve microbial safety and quality. Regulations in the United States require carcasses to be chilled to 4.4°C or lower in 4, 6 or 8 hours for carcasses weighing less than 4, 4 to 8, or over 8 pounds, respectively (28).

**(1) Immersion chilling:** Immersion chilling is done by holding carcasses in tanks containing ice (referred to as static chilling) or moving carcasses through a series of tanks containing chilled water, which more frequently contains ice (referred to as continuous immersion chilling). During continuous water immersion chilling, which is commonly used to reduce carcass temperature, carcasses pass through a series of cold water troughs. Large populations of campylobacters and some salmonellae are washed off carcasses during the agitation associated with continuous immersion chilling (108). In these common baths, some microorganisms float from the surfaces and away from the carcasses, whereas others redistribute to other carcasses. During immersion chilling the liquid film over carcasses is replaced by water from the chill tanks, and skin and collagenous connective tissues absorb water and swell (142). Factors that affect the reduction of microorganisms during continuous-immersion chilling include amount of bacterial contamination on the carcasses before chilling, amount of fresh water replacement per carcasses, and number of carcasses in relation to the volume of chiller water. If insufficient water is used per carcass, microorganisms can accumulate in the

water and increase on carcasses. Modern counter-flow immersion chilling procedures can improve carcass appearance by washing the outer surfaces, reduce the bacterial content on carcasses, minimize cross-contamination, and control organic matter in chill water that can reduce chlorine effectiveness.

**(2) Spray chilling:** Spray chilling consists of spraying chilled water onto carcasses as they are suspended in air. Large populations of microorganisms still remain after spray chilling. The large amount of water necessary for effective spray chilling has limited its use commercially. This system does not necessarily improve the hygienic state of poultry over other systems

**(3) Air chilling:** Air chilling is done with various combinations of temperature, humidity and time. When air chilling is used, birds are soft scalded at about 50°C to avoid skin discoloration due to dehydration. This scald temperature does little to reduce enteric microorganisms, so microbial quality of the air must be controlled to avoid carcass contamination. During dry air chilling, there is less contact among carcasses and no further interchange of surface fluids until packaging which rehydration occurs. Air chilling may be more effective than water chilling because of the drying effects, but the structure of poultry skin makes it difficult to dry completely, even after prolonged chilling (109). However, a study in the Netherlands showed that the processing of affected flocks resulted in contamination of the processing line no matter what type of chilling was used, either immersion or air chilling (54). There was also no difference in the shelf life of broilers at 0°C found as a consequence of the combined effects of scalding (50 and 57°C) and chilling method (air, water, and evaporative chilling) (102).



- ***Weighing, packaging, cutting-up, and other operations:*** Studies have revealed high rates of isolation of *C. jejuni* from water in the scald tank, feather and offal flumes, chiller and from equipment at various parts of processing line (6, 151). Equipment used for weighing, segregating, holding, transporting, cutting and other further processing operations plays a role in cross-contamination.

### **Microtopography of the skin of chicken carcasses and microbial attachment :**

Three general types of microorganisms constitute the microbial population of poultry carcasses: the natural flora of skin; the transient flora that happens to be on skin and feathers at the time of slaughtering; and contaminants to skin that are acquired during processing (59). Several mechanisms have been proposed to account for the attachment of bacteria on poultry carcasses (144). Contamination of poultry carcasses during processing may involve one or more of the following mechanisms (Thomas et. al., 1987: (a) *Retention*: Any process by which a population of bacteria remain in a liquid film on a surface after transient immersion in a bacterial suspension, (b) *Entrapment*: Maintenance of bacteria at a surface as a result of the microtopography of the surface rather than a particular attribute of the organism, (c) *Adsorption*: A physicochemical process by which organisms are sorbed to a solid-liquid interface by short-range attractive forces, and (d) *Adhesion*: Establishment of an intimate contact between the microbial cell and a surface. Adhesion may be specific or non-specific and may be mediated by polymer bridging (33) or organelles such as fimbriae and holdfasts (32).

During processing, retention occurs when carcasses come into contact with water containing bacteria. A film of water is retained on the carcass surface. Thus, the level of

contamination is related directly to the microbial concentration in the processing water (95). Rinsing carcasses with water containing a lower microbial population will reduce the microbial population that remains on the carcasses. Entrapment occurs when exposed tissue surfaces (e.g. skin, collagenous connective tissue layers of muscle) absorb water and begin to swell. Swelling exposes deep channels and crevices into which bacteria can penetrate and become entrapped (9, 85, 87, 141, 143). These bacteria cannot be removed by carcass sprays. They also would be protected to some degree from chemicals used for decontamination by the location in channels and crevices on the skin surfaces (141). The method used for scalding and defeathering determines the extent of physical damage to superficial layers of poultry skin. The greater the physical damage to epidermis and exposure of the dermal layer, the greater the risk of entrapment in and adhesion to the skin (72, 73, 74). It is also possible that the scalding operation opens feather follicles to aid feather removal, and the follicles remain open throughout processing until chilling where they close and entrap microorganisms. However, bacteria that are initially retained in the surface film of water may eventually become entrapped (86). Adhesion occurs when microorganisms adhere to surface tissues by binding to surface site with microbial surface components (e.g., capsules, slime layers, microfibrils, and fimbriae) (93). However, some research shows that microbial adhesion to carcasses during processing is not dependent upon flagella, fimbriae or electrostatic attraction (87, 119, 143). Lillard (1985) proposed that adherence occurred rapidly within 15s of exposure, but attachment is a time dependent process (83). McMeekin and Thomas (1978) reported that bacterial population but not time influenced attachment (95). Conner and Bilgili (1994) showed that there was no significant difference for attachment

concerning culture temperature (23 or 37°C), inoculum level (100 cells/skin, 1,000 cells/skin, or 10,000 cells/skin), or contact time (10 min, 20 min, or 30 min) (31). These conflicting results indicate that the nature of bacterial attachment to skin is complex and involves elusive factors.

While all three mechanisms (retention, entrapment, and adhesion) likely occur, the relative significance of each is uncertain (84, 144). Bacteria may become more closely associated with the outer skin surface as time elapses. This may be partially explained by entrapment and, perhaps, ‘non-specific’ adhesion. This more general form of adherence differs from the specific adhesion for salmonellae on fascia and loose connective tissue. The mechanism of *Salmonella* adhesion to poultry carcass occurs on the fascia or loose connective tissue that is under the skin and cover muscle (119). *Salmonella* appear to attach preferentially to connective tissue rather than to muscle fibres (9). Fascia is a loose meshwork of collagen and elastin fibres embedded in a matrix of glycosaminoglycans (GAG). Bacteria apparently adhere to the GAG matrix rather than to the collagen fibres as had been previously reported (145). Optimal conditions for adhesion involve neutral pH, very low ionic strength, prior immersion in water for a period of time (e.g. 20 minutes), and extended immersion in contact with the bacterial cells.

### **Chemical Sanitizers for Chicken Carcasses:**

#### ***Chlorine:***

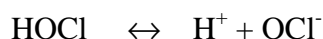
The chlorine used for disinfection is available in three forms: chlorine gas, liquefied compressed chlorine gas (chlorine water), sodium hypochlorite bleach solution,

and calcium hypochlorite pellet. In chlorination of water, a portion of the chlorine reacts with water impurities and the left over remains as residual available chlorine as referred to as free, combined, or total residual chlorine depending on the analytic method used. The term *free* available chlorine is usually applied to three forms of chlorine: (a) elemental chlorine ( $\text{Cl}_2$ ), (b) hypochlorous acid ( $\text{HOCl}$ ), and hypochlorite ion ( $\text{OCl}^-$ ) (150). Combined available chlorine refers to the combination of chlorine with ammonia or with other nitrogenous compounds in form of chloramines. Total residual (available) chlorine is free and combined available chlorine when present in water.

When chlorine reacts with water, a disproportion reaction with water occurs to form  $\text{HOCl}$  and  $\text{HCl}$ . The several possible forms of aqueous chlorine present in solution are elementary chlorine ( $\text{Cl}_2$ ), hypochlorous acid ( $\text{HOCl}$ ), hypochlorite ion ( $\text{OCl}^-$ ), chlorate ( $\text{ClO}_3^-$ ), chlorite ( $\text{ClO}_2^-$ ), chlorine dioxide ( $\text{ClO}_2$ ), and chloramine species, such as monochloramine, dichloramine, and trichloramine (49).

*Mechanism of action:*

$\text{HOCl}$ ,  $\text{OCl}^-$  and  $\text{Cl}_2$ , free available chlorine species, are considered as the most effective disinfective disinfection form of chlorine in addition to chlorine dioxide. When chlorine or hypochlorites are present in water, they undergo the following reaction:



Hypochlorous acid ( $\text{HClO}$ ) is the most effective chlorine species and much more effective than  $\text{OCl}^-$  as a disinfectant (107, 152). However, the forms of chlorine in aqueous solution depend on pH. At pH values below 4 to 5, nearly 100% of free chlorine

in solution is in the form of undissociated form of hypochlorous acid at 20°C, and at pH values above 10, nearly 100% of free chlorine in solution exists as hypochlorite (152). The  $pK_a$  value for HOCl is 7.5, which indicates that the concentrations of HOCl and  $OCl^-$  are equal at pH 7.5. Below pH 7.5, HOCl predominates and  $OCl^-$  dominates above pH 7.5. The effectiveness of chlorine in disinfection decreases with an increase in pH and a decrease in undissociated hypochlorous acid. High temperature and also organic materials, which are present in water, decrease bactericidal activity of chlorine (12, 81). The biocidal mechanisms of hypochlorite has not been clarified. It is believed that chlorine combines with proteins of cell membranes, forming *N*-chloro compounds which interfere with cell metabolism leading to cell death (5) and the oxidative reaction of chlorine on the sulfhydryl groups of vital enzymes or other enzymes sensitive to oxidation by chlorine (78).

### ***Hypochlorite:***

Liquid chlorine, hypochlorites, inorganic chloramines and organic chloramines, and chlorine dioxide are chlorine compounds that function as sanitizers. Hypochlorite is the most active among the chlorine compounds. Calcium hypochlorite and sodium hypochlorite are the major hypochlorite compounds. The effective form is in aqueous suspension, which requires a contact time of approximately 1.5 to 100 seconds (92). However, hypochlorites are available as powders or liquids. Calcium hypochlorite products are soluble in water and fairly stable on prolonged storage. The sodium hypochlorite in water solution is less stable. Because of their wide acceptance as

disinfectants in the food industry, hypochlorite solutions serve as standards for testing of other sanitizers and used as general sanitizers (40).

***Acidified Sodium Chlorite:***

Acidified sodium chlorite (ASC) is an FDA/USDA approved disinfectant, 21 CFR 173.325, produced by mixing an aqueous solution of sodium chlorite (CAS regulation number 7758-19-2) with a generally recognized as safe (GRAS) acid (147). Citric acid is used to activate ASC due to its effectiveness in killing *Campylobacter* over phosphoric acid (71). ASC is approved to use for the pre-chill or chiller tank. Sodium chlorite levels must be maintained between 50 and 150 ppm with a final pH concentration, with GRAS acid, of between 2.8 and 3.2 (147). As a spray or dip, sodium chlorite concentrations should be between 500 and 1200 ppm with GRAS acid levels high enough to produce a pH solution of 2.3 to 2.9.

***Mechanism of action:***

When  $\text{NaClO}_2$  reacts with organic matters, a number of organic intermediates are formed and cause a disruption of the oxidative bonds on the cell membrane surface. This makes it a broad-spectrum germicide (82). The commonly observed formation of resistant strains following prolonged exposure to antimicrobial is reduced due to the non-specific chemical reaction (71). Using a Food and Drug Administration defined test procedure (3), none of 10 test microbe strains formed any resistance to the chemical after 100 divisions of subinhibitory dose (71). Kemp et. al. (2000) reported using a 1200 ppm

spray of ASC on whole broiler carcasses to produce a reduction of 0.52, 0.77, and 0.52  $\log_{10}$  cfu/ml in aerobes, *E. coli*, and total coliform counts, respectively.

***Peracetic acid:***

Peracetic acid (PAA) is the peroxide of acetic acid, peroxyacetic acid. PAA is produced by the reaction of acetic acid or acetic anhydride with hydrogen peroxide in the presence of sulfuric acid, as a catalyst. To prevent the reverse reaction, the PAA solution is fortified with acetic acid, hydrogen peroxide, and stabilizer, which may be a sequestering agent (sodium pyrophosphate) or chelating agent. It is a more potent antimicrobial agent than hydrogen peroxide (7, 41). It has a rapid reaction at low concentration against a wide spectrum of microorganisms, remains effective in the presence of organic matter, and is not deactivated by catalase and peroxidases. As a weak acid, it is more active in acid condition but it is still germicidal at higher concentrations at alkaline pH values.

***Mechanism of action:***

There has been little investigation of the mechanism of action of PAA. PAA was shown to be one of the strongest bactericide, and that of the radicals formed the hydroxyl radical is the lethal species (29). Organic radicals formed from PAA, like  $\text{CH}_3\text{C}=\text{OO}\bullet$  and  $\text{CH}_3\text{C}=\text{O}\bullet$  might be involved in its sporicidal action due to their greater longevity than the hydroxyl radical (91, 124). The hydroxyl radical, being highly reactive, will attack membrane lipids, DNA, and other essential cell components. Sensitive sulfhydryl and sulfur bonds in enzymes, proteins, and other metabolites are oxidized by PAA. PAA

might also disrupt the osmotic function of the lipoprotein cytoplasmic membrane (18).

PAA inhibits and kills gram positive and negative bacteria, fungi and yeasts in 5 min or less at lower than 100 ppm.

The antimicrobial action of PAA at low temperatures along with the absence of toxic residuals has led to wide range of applications. PAA residues are acetic acid, oxygen, water, hydrogen peroxide, and dilute sulfuric acid. That makes it more acceptable for using in food processing industries. It has been accepted worldwide in the food processing and beverage industries, which include meat and poultry processing plants, canneries, dairies, breweries, wineries, and soft-drink plants (39).

PAA should be stored at cool temperatures in original containers for stability. It is unaffected by glass and most plastics. Pure aluminum, stainless steel, and tin-plated iron are resistant to PAA; but plain steel, galvanized iron, copper, brass, and bronze are susceptible to reaction and corrosion (121).

### **Microstructure studies using confocal scanning laser microscopy:**

#### ***Confocal scanning laser microscopy (CSLM):***

The confocal microscope is an improved version of the classical optical microscope. CSLM is a combination of traditional epifluorescence microscope hardware with a laser light source couple with specialized scanning equipment and computerized digital imaging. The word ‘confocal’ describes the optical coincidence of the illuminated and detection point (128). Optical paths of CSLM are designed so that when the laser beam is focused in the specimen, it will be confocal to the point of light focused at the pinhole in front of the photodetector. Then, only information from the focal plane of



interest reaches the photodetector. The laser provides the high degree of monochromaticity, small divergence, high brightness, high degree of spatial and temporal coherence, plane-polarized emission, and Gaussian beam profile that makes it an almost ideal light source for use in confocal microscopy (50). However, if the pinhole size is sufficiently large, illumination is no longer confocal, and the system behaves as a wide-field microscope (135).

In conventional light microscopy, noncoherent light is applied, and all the reflected light, including above and below the focal plane, is collected resulting in a blurred or diffused image for a specimen with considerable thickness (27). In contrast, in CSLM, a point light source (laser) is focused on a small volume within the specimen and only the result signal from a confocal light point is detected. This provides only an image of the in-focus plane with the out-of-focus parts appearing as black background (20). With this concept, not only the resolution is improved but also elimination of out-of-focus light allows observation of thick samples by enabling scanning in the z-axis without interference from light above and below the focal plane (20, 24, 113). Images of z series can be subsequently reconstituted using computer software to obtain three-dimensional (3-D) image or object of the original structure can be rotated or sliced in any direction (148). Other forms of microscopy such as transmission and scanning electron microscopy can improve the resolution significantly; however, the tedious and often harmful sample preparation becomes restriction to their applications. Specimens can be viewed “as-is” with the CSLM without prior dehydration steps, embedding, fixation, or sectioning.

Various modes, including reflectance and fluorescence, are available in most confocal systems. CSLM is favored over conventional fluorescence microscopy in that it offers a greater spatial resolution, with a signal-to-noise ratio (S/N) on the order of 100 times greater than that of the fluorescence microscope (120). CSLM also reduces photobleaching by illuminating only the focal point in the specimen, whereas with conventional epifluorescent microscopy, the entire specimen is excited (113). In addition, the detectors used in CSLM, such as photomultiplier tube and a charge-couple device (CCD), can detect low level signals, thus increase sensitivity of detection and more than one detector can be attached to the system so the simultaneous detection of multiple wavelength signals is available (113).

A limitation in using CSLM comparing to conventional microscopy and electron microscopy is that the laser used in CSLM produces light in limited narrow-wavelength bands, which are not available for excitation all fluorophores. Most commonly used is the argon-ion laser that gives a strong excitation at 488 nm, which is suitable for the excitation of fluorescein and its derivatives (113). A mixed-gas argon-krypton-ion laser provides lines at 488, 568, and 647 nm also used to analyze samples labeled with multiple dyes having different excitation and emission spectra.

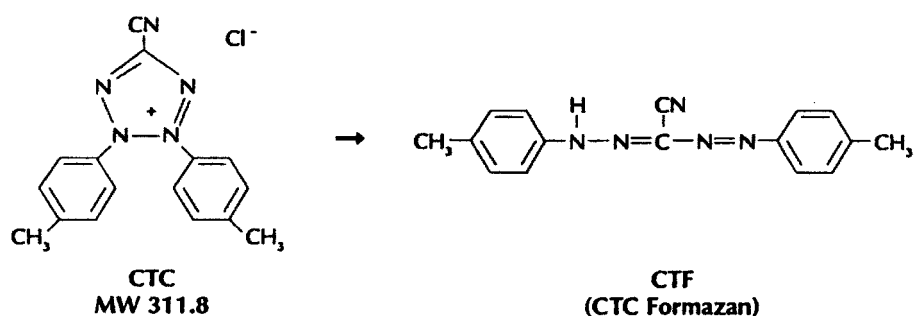
#### ***CSLM & the study of bacterial attachment to meat:***

CSLM is used in the biological sciences as a valuable tool for analyzing structure, physiology, and function of cells (19). In food safety aspect, the advantage of CSLM in observation of thick specimens without changing or destroying original structure as well as generating 3-D structure makes it suitable in study the attachment of bacteria. The

bacteria can be retained, entrapped, and attached on the meat surface. Several attachment studies have used SEM; however, chemical fixation and the dehydration process cause retained and entrapped cells to appear attached (135). Delaquis et. al. (1992) first observed spoilage microorganisms on porcine muscle stained with acridine orange (AO) using CSLM (36). Kim et. al. (1996) observed the attachment of *Salmonella* on chicken skin (75). The chicken skin and *Salmonella* were stained with FITC and Pyronin Y, respectively. *Salmonella* was found retaining in water film on chicken skin surface, in crevices and also entrapped in feather follicles. The ability of CSLM in observation in hydrated samples enabled one to locate bacteria in different locations.

### **Viability Staining - CTC (5-Cyano-2,3-ditolyl tetrazolium chloride)**

#### **Conformation:**



#### **History:**

The assessment of cell viability is one of the main requirements in several areas of microbiology, from environmental research to industrial applications. Unfortunately, no single analytical method can identify all physiological types of bacteria. Traditionally, plate count methods are used to detect cell viability, but they are time consuming and

require lengthy incubations and they typically do not provide useful information concerning physiological activity (or viability) in the absence of exogenous nutrient supplementation. In addition, plate count procedures cannot be used to directly observe active cells *in situ*, especially when the cells are attached to suspended particulate matter or other solid surface. Consequently, an alternative method, direct counting, was developed for bacterial enumeration.

Several direct techniques were developed for enumerating metabolically active bacteria. The method of Kogure et al. (77) involves incubation of a water sample amended with an assimilable carbon source in the presence of nalidixic acid, a specific DNA gyrase inhibitor that interferes with cell division in many gram negative bacteria . Growing cells that respond to nalidixic acid become elongated or swollen and can be directly enumerated microscopically to determine the actively growing subpopulation. However, the presence of filamentous or pleomorphic cells in certain water samples complicates interpretation of results obtained with this procedure. Furthermore, in the absence of an exogenously supplied carbon source, cell elongation may occur slowly or may be insufficient to measure accurately, precluding the application of this procedure in studies involving unamended samples.

Lundgren (1981) proposed a fluorescent method for directly determining the viability of soil bacteria based on the enzymatic hydrolysis of the fluorogenic ester fluorescein diacetate (89). However, many bacteria are evidently unable to transport fluorescein diacetate into the cell: the fluorescence emission tends to be weak, and background tends to be high because of autofluorescence (at similar emission wavelengths) of abiotic particles in many samples. Furthermore, the hydrolysis of

fluorescein diacetate is primarily a function of cellular esterase activity so it does not necessarily provide useful information concerning the respiratory status or growth potential of a bacterium.

Iturriaga (1979) employed the redox dye 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) to directly observe respirometrically active bacteria in aquatic environmental samples (62). The vital stain directly competes with molecular oxygen as an artificial electron acceptor. The reducing power generated by the electron transport system converts INT into insoluble INT-formazan crystals, which accumulate in metabolically active bacteria. Although the INT procedure has proved extremely useful in many environmental studies, it is difficult or impossible to visualize actively respiring cells on membrane filters and other surfaces that are optically opaque.

Some other ditetrazolium redox dyes that are similar to INT in molecular structure and function have been developed. However unlike INT, these new compounds produce fluorescent formazans when they are chemically or biologically reduced. One compound that has been successfully applied in cytochemical and histochemical studies is 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (122, 131, 132).

The 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is a monotetrazolium redox dye which produces a fluorescent formazan (CTF) when it is chemically or biologically reduced. The CTF is deposited intracellularly. Firstly, CTC had been employed as a cellular redox indicator of respiratory (i.e., electron transport) activity in cytochemical experiments with Ehrlich ascites tumor cells (122, 131, 132). Until 1992, Rodriguez et al. (117) described the first application of CTC for microscopic visualization of actively respiring bacteria in native and nutrient-amended environmental samples and in bacterial

biofilm formed on microscope slides. After that a variety of bacteria, including *Escherichia coli*, *Salmonella typhimurium* (88), *Campylobacter jejuni* (25, 42, 140) and *Yersinia enterocolitica*, have been observed to actively reduce CTC, as well as with environmental samples (154) and bacterial biofilm (146).

### **Characteristics:**

Generally, redox dyes have the ability to change color depending on their oxidative state. To be useful in both CSLM and flow cytometric studies, a redox dye would have to be fluorescent. CTC and the reduced formazan have ideal spectral properties, which has met these criteria. The reduced CTC does not absorb light above 400 nm, while the excitation max of the formazan is 450 nm and the emission max of the formazan is 630 nm. This is useful for application in both fluorescent microscopy and flow cytometric studies. Because CTC-formazan fluoresces primarily in the red region of the visible spectrum, it is readily distinguishable from most background fluorescence and autofluorescing abiotic particles, which typically emit in the blue or blue-green regions of the visible spectrum in natural water samples. The increased detectability of the fluorescent CTC-formazan facilitated enumeration of very small bacteria and bacteria exhibiting low electron transport activity with minimal deposition of the vital dye. In many bacteria, only a small ( $<0.2\ \mu\text{m}$ ) region of CTC-formazan deposition could be observed. In addition, CTC is water soluble in its oxidized state and water insoluble in the reduced form, which also facilitates its use for cell isolation or localization.

The CTC formazan is readily soluble in many polar organic solvents but is fluorescent only in the solid form, not in solution. The most useful solvents for CTC are

alcohols. Ethanol has been used frequently to elute the CTC formazan but dilute ethanol solutions (<50% ethanol) result in poor solubility. In previous work (122, 131, 132), CTC reduction was suppressed by specific electron transport inhibitors such as cyanide. It should be noted that CTC can be chemically reduced in low-redox environment (e.g. in the presence of sodium dithionate); hence, their use is restricted primarily to aerobic or microphilic systems (117). It is not clear whether CTC can be reduced by endogenous reductants in aerobic systems independently of biological electron transport. However, this possibility seems unlikely in Rodriguez's studies (1992), since formaldehyde and azide both suppressed CTC reduction and reduction tended to increase strongly upon the addition of oxidizable carbon substrates such as glucose or R2A broth.

Carbon supplementation (with R2A medium or glucose) is required to maximize the number of bacteria that can actively reduce CTC in the selected time frame (117). The CTC reduction activity of *Pseudomonas putida* at elevated reagent concentrations (> 6.0 mM) has been observed which may be from a toxic effect of the compound or possibly an impurity associated with the commercial product. However, Rodriguez (1992) suggested that 5.0 mM CTC maybe an optimal level of CTC for enumerating bacteria in environmental samples. Additional experiments will be needed to delineate more carefully the optimal reagent concentrations and to identify physiological and environmental factors that influence compound reduction.

#### **Application on *Campylobacter jejuni*:**

*C. jejuni* can be present in two morphological forms: culturable spiral forms and degenerating coccoid forms (25). Conveying from spiral to coccoidal form depends on

many factors (strains, temprature, pH, osmolarity, medium, etc) and requires variable time intervals. However, CTC can be used successfully for detection and studying of viable but non-culturable (VNC) bacterial state (25, 140). The presence of VNC cells poses a major public health problem since the traditional culturing methods cannot detect and the cells remain potentially pathogenic under favorable conditions.

Capelier et al (1997) compared the survival of *C. jejuni* by using the cellular elongation method (DVC) and the CTC procedure. Both methods were quite similar which confirms the validity of both for counting and detecting viable *C. jejuni* cells. However, they found that cell elongation for *C. jejuni* cells was very slow process (more than 7 h) and was not always easy to detect by microscopic observation because the transition to coccoid forms under stress made it more difficult to interpret than in bacillus (spiral) forms (25).

The quantification of respiring bacteria by CTC reduction is subject to experimental conditions, particularly for incubation times and CTC concentrations. CTC incubation time is crucial for time-limited uptake of CTC by viable but non-culturable cells. In addition, in case of microaerophilic bacteria, different time periods can be achieved in microaerobic environment. Coallier et al (1994) recommended longer incubation time (6-24 h) and lower CTC concentrations in order to avoid toxic effects and CTC impurities or deposits (30). However, physiological differences between bacterial groups influence the optimal incubation time needed to produce a maximum of CTC reducing cells. The optimal CTC concentration used also varies with experimental conditions. Mostly, 5 mM CTC (final concentration) is used, as recommended by Rodriguez et al (1992).



## REFERENCES

1. Acuff, G. R., C. Vanderzant, F. A. Gardner, and F. A. Golan. 1982. Examination of turkey eggs, poults and brooder houses facilities for *Campylobacter jejuni*. J. Food Prot. 45:1278-1281.
2. Anellis, A., D. Berkowitz, and D. Kemper. 1973. Comparative resistance of nonsporogenic bacteria to low-temperature gamma radiation. Appl. Microbiol. 25:517.
3. Anonymous. 1999. Testing of health-care antiseptic drug products. Code of Federal Regulation. Office of the Federal Register. 21CFR333.470(a)(1)(iii). Washington, D.C.: U. S. Government Printing Office.
4. Archer, D. L. 1988. The true impact of foodborne infections. Food Technol. 42:53-58.
5. Baker, J. C. 1926. Chlorine in sewage and waste disposal. Canadian Engineering, Water and Sewage. 50:127-128.
6. Baker, R. C., M. D. C. Paredes, and R. A. Qureshi. 1987. Prevalence of *Campylobacter jejuni* in poultry meat in New York State. Poult. Sci. 66:1766-1770.
7. Baldry, M. G. C. 1983. The bactericidal, fungicidal, and sporicidal properties of hydrogen peroxide and peracetic acid. J. Appl. Bacteriol. 54:417-423.
8. Beery, J. V., M. B. Hugdahl, and M. P. Doyle. 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl. Environ. Microbiol. 54:2365-2370.
9. Benedict, R. C., F. J. Schultz, and S. B. Jones. 1991. Attachment and removal of *Salmonella* spp. on meat and poultry tissues. J. Food Safety. 11:135-148.

10. 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.
11. Berrang, M. E., R. J. Buhr, J. A. Cason, and J. A. Dickens. 2001. Broiler Carcass Contamination with *Campylobacter* from Feces during Defeathering. *J. Food Prot.* 64(12):2063-2066.
12. Beuchat, L. R. 1998. Surface decontamination of fruits and vegetables eaten raw: a review. WHO/FSF/FOS/98.2. 42 p.
13. Black, R. E., M. M. Levine, M. L. Clements, P. Timothy. 1998. Experimental *Campylobacter jejuni* infections in humans. *J. Infect. Dis.* 157:472-479.
14. Blaser, M. J., I. D. Berkowitz, F. M. Laforce, J. Cravens, L. B. Reller, W-L. Wang. 1979. *Campylobacter* enteritidis: clinical and epidemiologic features. *Ann Intern Med.* 91:179-185.
15. Blaser, M. J. 1982. *Campylobacter jejuni* and food. *Food Technol.* 36:89-92.
16. Blaser, M. J., J. G. Wells, R. A. Feldman, R. A. Pollard, J. R. Allen, and the Collaborative Diarrheal Disease Study Group. 1983. *Campylobacter enteritis* in the United States. A multicenter study. *Ann. Intern. Med.* 98:360-365.
17. Blaser, M. J., D. N. Taylor, and R. A. Feldman. 1984. Epidemiology of *Campylobacter* infection. *In Campylobacter infection in man and animals.* Butzler. (eds). CRC Press. Boca Raton. p. 43-161
18. Block, S. S. 2001. Peroxygen Compounds. *In Disinfection, Sterilization, and Preservation.* S.S. Block. (eds). Lippincott Williams & Wilkins. Philadelphia, PA. p. 185-204
19. Boyde, A. 1994. Bibliography on confocal microscopy and its applications. *Scanning.* 16:35-36.

20. Brakenhoff, G. J., H. T. M. Van der Voort, E. A. Van Spronsem, and N. Nanninga. 1988. 3-Dimensional imaging of biological structures by high resolution confocal scanning laser microscopy. *Scanning. Microscopy.* 2(1):33-40.
21. Bryan, F. L. and M. P. Doyle. 1995. Health risks and consequences of *Salmonella* and *Campylobacter jejuni* in raw poultry. *J. Food Prot.* 58(3):326-344.
22. Butzler, J. P., P. Dekeyser, M. Detrain, and F. Dehaen. 1973. Related *Vibrio* in stools. *J. Pediatr.* 82:493-5.
23. Butzler, J. P. and M. D. Skirrow. 1979. *Campylobacter enteritis*. *Clin. Gastroentero.* 8:737-765.
24. Caldwell, D. E., D. R. Korber, and J. R. Lawrence. 1992. Confocal laser microscopy and digital image analysis in microbial ecology. *In Advances in microbial ecology.* K.C. Marshall (eds). Plenum Press. New York. p. 1-67
25. Cappelier, J. M., B. Lazaro, A. Rossero. A. Fernandez-Astorga, and M. Federighi. 1997. Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable *Campylobacter jejuni* cells. *Vet. Res.* 28:547-555.
26. Catteau, M. 1995. The genus *Campylobacter*. Microbiological control for foods and agricultural products. C.M. Bourgeois and J.Y. Leveau (eds). VCH Publishers, Inc. New York. 325-334.
27. Cavanagh, H. S., W. M. Petroll, and J. V. Jester. 1995. Confocal microscopy uses in measurement of cellular structure and function. *Progress in Retinal and Eye Res.* 14:527-565.
28. CFR. 1992. Temperatures and chilling and freezing requirements. General chilling requirement. Code of Federal regulations. Animals and Animal Products. 9CFR381.66b2. US Government Printing Office. Washington. 435 pp.
29. Clapp, P. A., M. J. Davies, and M. S. French. 1994. The bactericidal action of peroxides; on E. P.R. spin-trapping study. *Free Radic. Res.* 21:147-167.

30. Coallier, J., M. Prevost, and A. Rompre. 1994. The optimization and application of two direct viable count methods for bacteria in distributed drinking water. *Can. J. Microbiol.* 40:830-836.
31. Conner, D. E. and S. F. Bigili. 1994. Skin attachment model for improved laboratory evaluation of potential carcass disinfectants for their efficacy against *Salmonella* attached to broiler skin. *J. Food Prot.* 57(8): 684-688.
32. Corpe, W. A. 1980. Microbial surface components involved in adsorption of microorganisms onto surfaces. *In* Adsorption of microorganisms to surfaces. G. Bitton and K.C. Marshall (eds). Wiley Interscience. New York. p. 105-144
33. Costerton, J. W., G. G. Geesey, and K. J. Cheng. 1981. The role of bacterial surface structures in pathogenesis. *CRC Crit. Rev. Microbiol.* 8:303-338.
34. Deibel, K. E. and G. J. Banwart. 1984. Effect of spices on *Campylobacter jejuni* at three temperatures. *J. Food Safety.* 6:241-251.
35. Dekeyser, P. J., M. Goussuin-Detrain, J. P. Butzler, and J. Sternon. 1972. Acute enteritis due to related *Vibrio*: first positive stool cultures. *J. Infect Dis.* 125:390-392.
36. Delaquis, P. J., C. Gariepy, and D. Montpetit. 1992. Confocal scanning laser microscopy of porcine muscle colonized with meat spoilage bacteria. *Food Microbiol.* 9:147-153.
37. Deming, M. S., R. V. Tauxe, P. A. Blake, S. e. Dixon, B. S. Fowler, T. S. Jones, E. A. Lockamy, C. M. Patton, and R. O. Sikes. 1987. *Campylobacter* enteritis at a university: Transmission from eating chicken and from cats. *Am. J. Epidemiol.* 126(3):526-534.
38. Doyle, M. P. and D. J. Roman. 1981. Growth and Survival of *Campylobacter fetus* subsp. *jejuni* as a function of temperature and pH. *J. Food Prot.* 44(8):596-601.

39. Dychdala, G. R. 1988. New hydrogen peroxide-peroxyacetic acid disinfectant. *In* Proceedings of the 4th conference on progress in chemical disinfection. Binghamton, NY.
40. Dychdala, G.R. 2001. Chlorine and Chlorine Compounds. *In* Disinfection, Sterilization, and Preservation. S.S. Block (eds). Lippincott Williams & Wilkins. Philadelphia, PA. p. 135-157
41. Eggensberger, H. 1979. Disinfectants based on peracid splitting compounds. *Zentralbl Bakteriol Mikrobiol Hyg [B]*. 168:517-524.
42. Federighi, M., J. L. Tholozan, J. M. Cappelletti, J. P. Tissier, and J. L. Jouve. 1998. Evidence of non-coccoid viable but non-culturable *Campylobacter jejuni* cells in microcosm water by direct viable count, CTC-DAPI double staining, and scanning electron microscopy. *Food Microbiology*. 15:539-550.
43. Finch, M. J. and P. A. Blake. 1985. Foodborne outbreaks of campylobacteriosis: The United States experience, 1980-1982. *Am. J. Epidemiol.* 122(2):262-268.
44. Genigeorgis, C., M. Hassane, and P. Collins. 1986. *Campylobacter jejuni* infection on poultry farms and its effect on poultry meat contamination during slaughter. *J. Food Prot.* 49(11):895-903.
45. George, H. A., P. S. Hoffman, R. M. Smibert, and N. R. Kreig. 1978. Improved media for growth and aerotolerant of *Campylobacter fetus*. *J. Clin. Microbiol.* 8:36-41.
46. Georges-Courbot, M. C., C. Banya, A. M. Beraud, D. M. Y. Meurier, and A. J. Georges. 1986. Distribution and serotypes of *Campylobacter jejuni* and *Campylobacter coli* in enteric *Campylobacter* strains isolated from children in the Central African Republic. *J Clin Microbiol.* 23:592-594.
47. Gill, C. O. and L. M. Harris. 1983. Limiting conditions of temperature and pH for growth of "thermophilic" campylobacters on solid media. *J. Food Prot.* 46:767-768.

48. Glass, R. I., B. J. Stoll, M. I. Huq, M. J. Struelens, M. J. Blaser, and A. K. G. Kibriya. 1983. Epidemiology and clinical features of endemic *Campylobacter jejuni* infection in Bangladesh. *J. Infect Dis.* 148:292-296.
49. Gordon, G., W. J. Cooper, R. G. Rice, and G. E. Pacey. 1992. Chlorine, chloramines, and the oxy-chlorine species. *In* Disinfectant Residual Measurement Methods. AWWA Research Foundation and American Water Works Association. p. 11-62
50. Gratton, E. and M. J. VandeVen. 1995. Laser Sources for Confocal Microscopy. *In* Handbook of Biological Confocal Microscopy. J.B. Pawley (eds). Plenum Press. New York. p. 69-97
51. Grau, F. H. 1986. Microbial ecology of meat and poultry. *In* Advances in meat research. A.M. Pearson and T.R. Dutson (eds). AVI publ. Co. Westport, CT. p. 1-47
52. Gutman, L. T. 1992. *Campylobacter*, *Helicobacter*, and *Spirillum*. *In* Zinsser Microbiology. W.K. Joklik, H. P. Willett, D. B. Amos, and C. M. Wilfert (eds). Appleton & Lange. Norwalk, Connecticut.
53. Harris, N. V., N. S. Weiss, and C. M. Nolan. 1986. The role of poultry and meats in the etiology of *Campylobacter jejuni/coli enteritis*. *Am. J. Public Health.* 76:407-411.
54. Hartog, B. J., G. J. A. Dewild, and E. DeBeor. 1983. Poultry as a source of *Campylobacter jejuni*. *Arch. Lebensmittelhyg.* 34:116-122.
55. Hoffman, P. S., H. A. George, N. R. Kreig, and R. M. Smibert. 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. II Role of exogenous superoxide anions and hydrogen peroxide. *Can. J. Microbiol.* 25:8-16.
56. Hoffman, P. S., N. R. Kreig, and R. M. Smibert. 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. I. Physiological aspects of enhanced aerotolerance. *Can. J. Microbiol.* 25:1-7.

57. Hugdahl, M. B., J. T. Berry, and M. P. Doyle. 1988. Chemotactic behavior of *Campylobacter jejuni*. *Infect. Immun.* 56:1560-1566.
58. Humphrey, C. D., D. M. Montag, and F. E. Pittman. 1986. Morphologic observation of experimental *Campylobacter jejuni* infection in the hamster intestinal tract. *Am. J. Pathol.* 122:152-159.
59. ICMSF. 1998. Poultry and poultry products. *In* Microorganisms in Foods 6: Microbial Ecology of Food Commodities. ICMSF (eds). Blackie Academic & Professional. New York. p. 75-129
60. Ingram, M. and A.C. Kitchell. 1967. Salt as a preservative for foods. *J. Food Technol.* 2:1-15.
61. International Commission on Microbiological Specifications for Foods. 1980. Food commodities. *In*. Microbial Ecology of Foods. Vol. 2. Academic Press. New York, NY.
62. Iturriaga, R. 1979. Bacterial activity related to sedimenting particulate matter. *Mar. Biol.* 55:157-169.
63. Izat, A.L., F. A. Gardner, J. H. Denton, and F. A. Golan. 1988. Incidence and level of *Campylobacter jejuni* in broiler processing. *Poult. Sci.* 67:1568-1572.
64. Jacobs-Reitsma, W. 2000. *Campylobacter* in the food supply. *In* Campylobacter. I. Nachamkin and M.J. Blaser (eds). ASM Press. Washington, DC. p. 467-481
65. Jones, F. T., R. C. Axtell, D. V. Rives, S. E. Scheideler, F. R. Tarver, Jr., R. L. Walker, and M. J. Wineland. 1991. A survey of *Campylobacter jejuni* contamination in modern broiler production and processing systems. *J. Food Prot.* 54(4):259-262.
66. Jul, M. 1986. Chilling broiler chicken, an overview. *In* Meat Chilling. Paris: International Institute of Refrigeration.

67. Kaldor, J. and B. R. Speed. 1984. Guillian-Baire syndrome and *Campylobacter jejuni*: a serological study. Br. Med. J. 288:1867-1870.
68. Karmali, M. A. and P. C. Flemming. 1979. *Campylobacter* enteritidis. Can. Med. Assoc. J. 120:1525.
69. Kasrazadeh, M. and C. Genigeorgis. 1987. Origin and prevalence of *Campylobacter jejuni* in ducks and duck meat at the farm and processing plant level. J. Food Prot. 50:321-326.
70. Kazwala, R. R., J. D. Collins, J. Hannan, R. A. R. Crinion, and H. O'Haluny. 1990. Factors responsible for the introduction and spread of *Campylobacter jejuni* in chicken wings. Vet. Rec. 126:305-306.
71. Kemp, G., M. L. Aldrich, and A. L. Waldroup. 2000. Acidified sodium chlorite antimicrobial treatment of broiler carcasses. J. Food Prot. 63:1087-1092.
72. Kim, J.-W., S. J. Knabel, and S. Doores. 1993. Penetration of *Salmonella typhimurium* into turkey skin. J. Food Prot. 56:292-296.
73. Kim, J.-W. and S. Doores. 1993. Influence of three defeathering systems on microtopography of turkey skin and adhesion of *Salmonella typhimurium*. J. Food Prot. 56:286-291.
74. Kim, J.-W. and S. Doores. 1993. Attachment of *Salmonella typhimurium* to skins of turkey that had been defeathered through three different systems, scanning electron microscopic examination. J. Food Prot. 56:395-400.
75. Kim, K. Y., J. F. Frank, and S. E. Craven. 1996. Three-dimensional visualization of *Salmonella* attachment to poultry skin using confocal scanning laser microscopy. Lett. Appl. Microbiol. 22:280-282.
76. King, E. O. 1962. The laboratory recognition of *Vibrio fetus* and closely related *Vibrio* species isolated from cases of human vibriosis. Ann NY Acad Sci. 98:700-711.



77. King, L. A. and B. C. Parker. 1988. A simple, rapid method for enumeration total viable and metabolically active bacteria in groundwater. *Appl. Environ. microbiol.* 46:1630-1631.
78. Knox, W. E., P. K. Stumpf, and D. E. Green. 1948. The inhibition of sulfhydryl enzymes as the basis of the bactericidal action of chlorine. *J. Bacteriol.* 55:451-458.
79. Konkel, M. E., D. J. Mead, S. F. Hayes, and W. Cieplak, Jr. 1992. Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures. *J. Infect. Dis.* 166:308-315.
80. Kotula, K. L. and Y. Pandya. 1995. Bacterial Contamination of Broiler Chickens before Scalding. *J. Food Prot.* 58(12):1326-1329.
81. Kotula, K. L., A. W. Kotula, B. E. Rose, C. J. Pierson, and M. Camp. 1997. Reduction of aqueous chlorine by organic material. *J. Food Prot.* 60:276-282.
82. Kross, R. 1984. An innovative demand-release microbiocide. Second biannual conference on progress in chemical disinfection. SUNY. Binghamton, New York.
83. Lillard, H. S. 1985. Bacterial cell characteristics and conditions influencing their adhesion to poultry skin. *J. Food Prot.* 98:803-807.
84. Lillard, H. S. 1986. Distribution of 'attached' *Salmonella typhimurium* cells between poultry skin and a surface film following water immersion. *J. Food Prot.* 49:449-454.
85. Lillard, H. S. 1988. Effect of surfactant or changes in ionic strength on the attachment of *Salmonella typhimurium* to poultry skin and muscle. *J. Food Sci.* 53:727-730.
86. Lillard, H. S. 1989. Incidence and recovery of salmonellae and other bacteria from commercially processed poultry carcasses at selected pre- and post-evisceration steps. *J. Food Prot.* 52:88-91.

87. Lillard, H. S. 1989. Factors affecting the persistence of *Salmonella* during the processing of poultry. *J. Food Prot.* 52:829-832.
88. Lopez-Amoros, R., S. Castel, J. Comas-Riu, and J. Vives-Rego. 1997. Assessment of *E. coli* and *Salmonella* viability and starvation by confocal laser microscopy and flow cytometry using rhodamine 123, DiBAC4(3), propidium iodide and CTC. *Cytometry.* 29:298-305.
89. Lundgren, B. 1981. Fluorescein diacetate as a stain for metabolically active bacteria. *Oikos.* 36:17-22.
90. Mahon, C. R. and G. Manuselis. 2000. Textbook of diagnostic microbiology. W.B. Saunders Company. Philadelphia, PA.
91. Marquis, R. E., G. C. Rutherford, and M. M. Faraci. 1995. Sporocidal action of peracetic acid and protective effects of transition metal ions. *J. Industrial Microbiology.* 15:486-492.
92. Marriott, N. G. 1999. Principles of Food Sanitation. 4th ed. Aspen Publ., Inc. Maryland. p. 139-157.
93. Marshall, K. C. and G. Bitton. 1980. Microbial Adhesion in Perspective. *In* Adsorption of microorganisms to surfaces. G. Bitton and K.C. Marshall (eds). A Wiley-Interscience Publication. New York. p. 1-5
94. May, K. N. 1961. Skin contamination of broilers during commercial evisceration. *Poult. Sci.* 40:531-536.
95. McMeekin, T. A. and C. J. Thomas. 1978. Retention of bacteria on chicken skin after immersion in bacterial suspensions. *J. Appl. Bacteriol.* 45:383-387.
96. Mead, G. C. 1982. Microbiology of the poultry and game birds. *In* Meat Microbiology. M.H. Brown (eds). Applied Science Publ. London. p. 67-101

97. Mead, G. C., W. R. Hudson, and M. H. Hinton. 1993. Microbiological survey of five poultry processing plants in the UK. *British Poultry Science*. 34:497-503.
98. 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. Infect.* 115:495-500.
99. Mehle, J., M. Gubin, and B. Gliha. 1981. Contamination of chicken meat with *Campylobacter jejuni* during the process of industrial slaughter. *In* Proceeding International workshop on *Campylobacter* Infections held at Univ. of Reading. MTP Press. Lancaster, England.
100. Mulder, R. W. A. W., L. W. J. Dorrestelin, and J. Van der Broek. 1978. Cross-contamination during the scalding and plucking of broiler. *Br. Poult. Sci.* 19:61-70.
101. Mulder, R. W. A. W. 1985. Decreasing microbial contamination during poultry processing. *Poultry*. March:52-55.
102. Mulder, R.W.A.W. and N. M. Bolder. 1987. Shelf-life of chilled poultry after various scalding and chilling treatments. *Fleischwirtschaft*. 67:114-116.
103. Nachamkin, I. 1997. *Campylobacter jejuni*. *In* Food Microbiology Fundamentals and Frontiers. M. P. Doyle, L. R. Beuchat, and T. J. Montville. (eds). ASM Press. Washington D.C. p. 159-170
104. Newell, D. G. and A. D. Pearson. 1981. Pathogenicity of *Campylobacter jejuni* - an *in vitro* model of adhesion and invasion? *In* *Campylobacter* Epidemiology, Pathogenesis and Biochemistry. D.G. Newell (eds). MTP Press. Hingham, MA.
105. Notermans, S. and E. H. Kampelmacher. 1975. Further studies on attachment of bacteria to skin. *Br. Poult. Sci.* 16:487-496.
106. Notermans, S., F. M. Van Leusden, and M. Van Schothorst. 1977. Suitability of different bacterial groups for determining faecal contamination during post scalding stages in the processing of broiler chickens. *J. Appl. Bacteriol.* 43:383-389.

107. Nunes, M. C. N. and J.-P. Edmond. 1999. Chlorinated water treatments affect postharvest quality of green bell peppers. *J. Food Qual.* 22(3):353-361.
108. Oosterom, J., S. Notermans, H. Karman, and G. B. engels. 1983. Origin and prevalence of *Campylobacter jejuni* in poultry processing. *J. Food Prot.* 46:339-344.
109. Oosterom, J., G. J. A. De wilde, E. De Boer, L. H. De Blaauw, and H. Karman. 1983. Survival of *Campylobacter jejuni* during poultry processing and pig slaughtering. *J. Food Prot.* 46:702-706, 709.
110. Park, R. W., P. L. Griffiths, and G. S. Moreno. 1991. Sources and survival of campylobacters: relevance to enteritis and the food industry. *J. Appl. Bacteriol.* 20(Suppl.):97S-106S.
111. Patterson, J. T. 1973. Airborne microorganisms in poultry processing plants. *Br. Poult. Sci.* 14:161-165.
112. Patterson, M. F. 1995. Sensitivity of *Campylobacter* spp. to irradiation in poultry meat. *Lett. Appl. Microbiol.* 20:338-340.
113. Pawley, J. B. 1995. Handbook of biological confocal microscopy. Plenum Press. New York.
114. Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell. 1993. Colonization of broiler chickens by waterborne *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 59:987-996.
115. Prescott, J. F. and O. S. Gellner. 1984. Intestinal carriage of *Campylobacter jejuni* and *Salmonella* by chicken flocks at slaughter. *Can. J. Comp. Med.* 48:329-331.
116. Robertson, D. A. 1981. Infective dose of *Campylobacter jejuni* in milk. *Br. Med. J.* 1:1594.

117. Rodriguez, G. G., D. Phipps, K. Ishiguro, and H. F. Ridgway. 1992. Use of a Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bacteria. *Appl. Environ. Microbiol.* June:1801-1808.
118. Rosef, O., B. Gondrosen, and G. Kapperud. 1984. *Campylobacter jejuni* and *Campylobacter coli* as surface contaminants of fresh and frozen poultry carcasses. *Int. J. Food Microbiol.* 1:205-215.
119. Sanderson, K., C. J. Thomas, and T. A. McMeekin. 1991. Molecular basis of the adhesion of *Salmonella* serotypes to chicken muscle fascia. *Biofouling.* 5:89-101.
120. Sandison, D. R. and W. W. Webb. 1994. Background rejection and signal-to-noise optimization in confocal and alternative fluorescence microscopes. *Appl. Optics.* 33:603-615.
121. Schroder, W. 1984. Peracetic acid: disinfectant for food industry. Nurnberg: Brauwelt International. p. 115-120.
122. Severin, E., J. Stellmach, and H. Nachtigal. 1985. Fluorimetric assay of redox activity in cells. *Anal. Chim. Acta.* 170:341-346.
123. Shane, S. M. 1991. Environmental factors associated with *Campylobacter jejuni* colonization of poultry. In *Colonization Control of Human Bacterial Enteropathogen in Poultry*. L.C. Blankenship (eds). Academic Press. San Diego, CA. p. 29-46
124. Shin, S. Y., E. G. Calvisi, and T. C. Beaman. 1994. Microscopic and thermal characterization of hydrogen peroxide killing and lysing of spores and protection by transition metal ions, chelators, and antioxidants. *Appl. Environ. Microbiol.* 60:3192-3197.
125. Simonsen, B. 1975. Microbiological aspects of poultry meat quality. Quality of Poultry Meat. In *Proceedings European Symposium on Poultry Meat Quality* (eds). Oosterbeek. The Netherlands. p. 1-10

126. Skirrow, M. B. 1977. *Campylobacter* enteritis: a "new" disease. Br. Med. J. 2:9-11.
127. Skirrow, M. B. 1990. *Campylobacter*. Lancet. 336:921-923.
128. Slavik, J. 1998. Single and multiple spectral parameter fluorescence microscopy. In Digital image analysis of microbes. M.H.F. Wilkinson and F. Schut (eds). John Wiley & Sons. New York. p. 93-114
129. Smibert, R. M. 1984. *Campylobacter*. In Bergey's manual of systematic bacteriology. N. R. Krieg and J. G. Holt (eds). Williams & Wilkins. Baltimore.
130. Smitherman, R. E., C. A. Genigeorgis, and A. Farver. 1984. Preliminary observations on the occurrence of *Campylobacter jejuni* at for California chicken ranches. J. Food Prot. 47:293-298.
131. Stellmach, J. 1984. Fluorescent redox dyes. I. Production of fluorescent formazan by unstimulated and phorbol ester or digitonin-stimulated Ehrlich ascites tumor cells. Histochem. 80:137-143.
132. Stellmach, J. and E. Severin. 1987. A fluorescent redox dye: influence of several substrates and electron carriers on the tetrazolium salt-formazan reaction of Ehrlich ascites tumor cells. Histochem. 19:21-26.
133. Stern, N.J., M. R. S. Clavero, J. S. Bailey, N. A. Cox, and M. C. Robach. *Campylobacter* spp. in Broilers on the Farm and After Transport. Poult. Sci. 74:937-941.
134. Szymanski, C. M., M. King, M. Haardt, and G. D. Asrmstrong. 1995. *Campylobacter jejuni* motility and invasion of Caco-2 cells. Infect. Immun. 63:4295-4300.
135. Takeuchi, K. and J. F. Frank. 2001. Confocal microscopy and microbial viability detection for food research. J. Food Prot. 64(12):2088-2102.

136. Tarjan, V. 1981. Investigation of the radiosensitivity of *Campylobacter fetus* subspecies *jejuni* in ground chicken meat. *Int. J. Food Microbiol.* 1:321-326.
137. Tauxe, R. V. 1992. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. *In Campylobacter jejuni: Current status and future trends.* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (eds). American Society for Microbiology. Washington, D. C.
138. Taylor, D. N., P. Echeverria, C. Pitarangsi, J. Seriwatana, L. Bodhidatta, and M. Blaser. 1988. Influence of strain characteristics and immunity on the epidemiology of *Campylobacter* infections in Thailand. *J. Clin Microbiol.* 26:863-868.
139. The National Advisory Committee on Microbiological Criteria for Foods. 1994. *Campylobacter jejuni/coli.* *J. Food Prot.* 57:1101-1121.
140. Tholozan, J. L., J. M. Cappelier, J. P. Tissier, G. Delattre, and M. Federighi. 1999. physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. *Appl. Environ. Microbiol.* 65(3):1110-1116.
141. Thomas, C. J. and T. A. McMeekin. 1980. Contamination of broiler carcass skin during commercial processing procedures, an electron microscopic study. *Appl. Environ. Microbiol.* 40:133-144.
142. Thomas, C. J. and T. A. McMeekin. 1982. Effect of water immersion on the microtopography of the skin of chicken carcasses. *J. Sci. Food Agri.* 33:549-554.
143. Thomas, C. J. and T. A. McMeekin. 1984. Effect of water uptake by poultry tissue on contamination by bacteria during immersion in bacterial immersions. *J. Food Prot.* 47:398-402.
144. Thomas, C. J., T. A. McMeekin, and J. T. Patterson. 1987. Prevention of microbial contamination in the poultry processing plant. *In Elimination of pathogenic organisms from meat and poultry.* F.J.M. Smulders (eds). Elsevier Science Publ. Amsterdam, The Netherlands. p. 163-179

145. Thomas, C. J. and T. A. McMeekin. 1991. Factors which affect retention of *Salmonella* by chicken muscle fascia. *Biofouling*. 5:75-87.
146. Trachoo, N. 2002. Survival of *Campylobacter jejuni* in biofilms isolated from water system of a chicken house. *J. Food Prot.* 65(7):1110-1116.
147. United States Department of Agriculture. 1982. Acidified sodium chlorite. 9 CFR 173.325(a) Office of the Federal Register. U. S. Government Printing Office. Washington, D.C.
148. Vodovotz, Y., E. Vittadini, J. Coupland, D. J. McClements, and P. Chinachoti. 1996. Bridging the Gap: use of confocal microscopy in food research. *Food Tech.* 74-82.
149. Wang, W. L., B. W. Powers, N. W. Luechterfeld, and M. J. Blaser. 1983. Effects of disinfectant on *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 45(4):1202-1205.
150. Weidenkopf, S. J. 1953. Water chlorination. *U. S. Armed Forces Medical Journal.* 4:253-261.
151. Wempe, J. M., C. A. Genigerorgis, T. B. Farver, and H. I. Yusufu. 1983. Prevalence of *Campylobacter jejuni* in two California chicken processing plants. *Appl. Environ. Microbiol.* 45:355-359.
152. White, G. C. 1999. Chemistry of Chlorination. *Handbook of chlorination & alternative disinfectant*. 4th ed. John Wiley & Sons, Inc. New York. p. 212-287.
153. Winer, J.B., R. A. C. Hughes, M. J. Anderson, D. M. Jones, H. Kangro, and R. P. F. Watkins. 1988. A retrospective study of acute idiopathic neuropathy. II. Antecedent events. *J. Neurol. Neurosurg. Psychiatry.* 51:613-618.
154. Yu, W., W. K. Dodds, M. K. Blanks, J. Skalsky, and E. A. Strauss. 1995. Optimal Staining and Sample Storage Time for Direct Microscopic Enumeration of Total and Active Bacteria in Soil with Two Fluorescent Dyes. *Appl. and Environ. Microbiol.* :3367-3372.



155. Yusufu, H.O., C. Genigeorgis, T. B. Farver, J. M. Wempe. 1983. Prevalence of *Campylobacter jejuni* at different sampling sites in two California turkey processing plants. J. Food Prot. 46:868-872.

## CHAPTER 1

### DIRECT MICROSCOPIC OBSERVATION OF *CAMPYLOBACTER JEJUNI* VIABILITY ON CHICKEN SKIN<sup>1</sup>

---

<sup>1</sup>Chantarapanont, W., M. Berrang, and J. F. Frank. 2002. To be submitted to Journal of Food Protection

## ABSTRACT

The objective of this study was to develop a method to identify specific sites on chicken skin, which allow survival of *Campylobacter jejuni*. This method employs confocal laser scanning laser microscopy (CSLM) visualization of *Campylobacter jejuni* transformed with P<sub>c</sub>*gfp* plasmid (GFP-*Campylobacter*) exposed to 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a redox dye which when taken up is reduced to CTC-formazan, and accumulated intracellularly in respiratory active cells. An Ar laser (excitation wavelength [ $\lambda$ ] = 488 nm) was used to excite both GFP-*Campylobacter* and CTC. The reflected light (483-495 nm) was assigned as a grey color for chicken skin-reflected light, emitted light (495-540 nm) as green color for GFP-*Campylobacter* image, and 600-670 nm emission as red color for CTC image. After 1 h inoculation with 2 ml of  $10^8$ - $10^9$  cfu/ml GFP-*Campylobacter* suspension,  $10^5$ - $10^6$  cfu of *C. jejuni* remained on 1 cm<sup>2</sup> of chicken breast skin after rinsing. Green fluorescence of all *C. jejuni* cells as well as the CTC-formazan in viable *Campylobacter* was clearly visible on chicken skin. The data indicated that GFP-*Campylobacter* remaining on the chicken skin surface after rinsing were mostly located in crevices, entrapped inside feather follicles with water and also entrapped in the surface water layer. Most viable cells were entrapped with water in the skin crevices and feather follicles. These sites provide a suitable microenvironment for GFP-*Campylobacter* to survive.

Relative to other foodborne pathogens *Campylobacter fetus* subsp. *jejuni* or *C. jejuni* caused the highest incidence of outbreaks during 1996-2001 (CDC, 2002).

Campylobacters are part of the normal flora in the intestinal tract of a large number of wild and domesticated animals including food animals, chickens and swine (Blankenship and Craven, 1982 and Lee et al, 1998). *C. jejuni* has been detected on raw chicken carcasses, and nearly all retail broiler chickens are contaminated with this bacterium (Stern, 1992a).

*Campylobacter* are slender, gram-negative, motile, helically curved rods 0.2 to 0.5  $\mu\text{m}$  wide and 0.5 to 5  $\mu\text{m}$  long. They are microaerophilic and require a low oxygen tension (3 to 15 percent) and an increased  $\text{CO}_2$  level (3 to 5 percent) for growth.

Atmospheric oxygen and other stresses commonly encountered in food and the environment are toxic to it. However, the microorganism can survive on chicken carcasses during refrigerated transport and frozen storage (Simmons and Gibbs, 1979).

Determining the effect of attachment to chicken skin on viability of *Campylobacter* will aid in understanding its survival in this environment and transmission to humans. Previous studies on attachment of pathogenic bacteria to poultry skin involved *Salmonella* and used indirect immunofluorescence (Kim et al, 1993a and Kim et al, 1996c), electron microscopy (Kim et al, 1993a and b), and radiolabeling (Kim et al, 1996b). While these methods successfully detect adherent bacteria, they have limited ability to specifically detect viable cells (Miller et al., 2000).

Using a fluorescent antibody to identify *Campylobacter* is difficult due to the lack of antibody specificity and the presence of nonspecific fluorescent background (Buswell et al, 1998). Bacteria with an intrinsic tag that can be detected in real time have many

advantages for studying adherence (Miller et al., 2000). The green fluorescent protein (GFP) of *Aequorea victoria*, encoded by the *gfp* gene, fluoresces without any added cofactor or substrate and can be expressed in a wide variety of bacterial species. Miller et al. (2000) demonstrated that GFP in *Campylobacter* is very stable and resistant to photobleaching. The intrinsic fluorescence and stability of GFP permit the nondestructive visualization of *gfp*-containing cells, even in complex environments.

Bacterial cell viability can be detected by staining with CTC (5-cyano-2,3-ditolyl tetrazolium chloride). CTC is a monotetrazolium redox dye which produces a fluorescent formazan (CTF) when it is chemically or biologically reduced. The CTF is deposited intracellularly. CTC was first employed as a cellular redox indicator of active respiratory (i.e., electron transport) in cytochemical experiments with Ehrlich ascites tumor cells (Stellmach, 1984). It has also been used for detecting viable but non-culturable *C. jejuni* (Cappelier, 1997, Federighi et al., 1998, and Tholozan et al., 1999). Among redox dyes, CTC is suitable for epifluorescent microscopy because CTC-formazan fluoresces primarily in the red region of the visible spectrum range making it readily distinguishable from most background fluorescence, which typically emits in the blue or blue-green regions of the spectrum. The sensitivity of detection of fluorescent CTC-formazan facilitates visualization of small bacteria and bacteria exhibiting low electron transport activity.

The objective of this study was to develop a method to identify specific sites on chicken skin including the surface and also deeper in feather follicles and channels, which allow *Campylobacter jejuni* attachment and survival. Confocal scanning laser microscopy was employed to visualize *gfp* *Campylobacter* incubated with CTC.

## MATERIALS AND METHODS

### **Bacterial strain and plasmid:** GFP *Campylobacter jejuni* RM 1221

(pWM1007) was provided by W. G. Miller USDA Agricultural Research Service, Albany, CA. The plasmid encoding GFP (pWM1007; Km<sup>r</sup>;pMW10 $\Delta$ lacZ $\Omega$ [(T1)<sub>4</sub>-P<sub>c</sub>-gfp-T1] had been mobilized into *C. jejuni* strain RM 1221 from a chicken carcass isolate (Miller et al., 2000). Transformants were selected by plating on Cefex-campy agar (CCA) (Stern, 1992b) amended with 200  $\mu$ g of kanamycin per ml (CCA-KM) and grown at 42°C for 48 hr in a sealed bag with a mixture of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub> (BOC Gases, Chattanooga, TN). Colonies on CCA-KM were restreaked on fresh plates, and expression of GFP-*Campylobacter* was confirmed by observation of bacterial smears with a Nikon Lobophot epifluorescent microscope (Nikon, Inc., Garden city, N.Y.) equipped with an 100x objective lens, 100-W Hg lamp, and XF19 filter cube (excitation filter = 455DF70; dichroic filter = 505DCLPEXT02; emission filter = 515EFLP) (Omega Optical Inc., Brattleboro, Vt.) and a Kodak DC120 zoom digital camera (Eastman Kodak Company, Rochester, N.Y.) Kanamycin- resistant clones, which exhibited green fluorescent by microscopic observation were stored at -70°C in 0.1% phosphate buffered saline (PBS) with sterile 20% glycerol.

**Culture Preparation:** Stock cultures of GFP-*Campylobacter* were resuscitated by streaking on CCA-KM (Stern et al, 1992b) and incubated at 42°C for 48 hr in a sealed bag containing of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>. Cultures were then transferred at least twice before using. For the attachment assay, a loopful of isolated colonies of GFP-

*Campylobacter* on CCA-KM agar (at the third and fourth quarter of streaking pattern) was suspended in 2 ml sterile deionized water (SDW). The optical density (O.D.) of the cell suspension was adjusted to 0.4 – 0.5 using a spectrophotometer (Spectronic 21D; Milton Roy Co., Rochester, NY) at 540 nm to yield  $10^8$  -  $10^9$  cells/ml.

**Chicken skin:** Freshly processed broiler carcasses were randomly collected immediately upon exiting the chill tank in a commercial processing plant. Carcasses were individually bagged and stored in ice no more than 30 min before removal of breast skins. Breast skin (6 x 6 cm) was removed from the same location on breasts of broiler carcasses for each assay using sterile scalpel and forceps. Chicken skin was aseptically stored at -20°C, no more than one week before using.

**Attachment assay:** Attachment was determined as described by Kim et al (1996c). Frozen chicken skin was thawed by rinsing 3 times for 3 min each with 15 ml of 4°C SDW on an orbital shaker model No. 3520 (Lab Line Instrument Inc, IL) at 100 rpm. Skin was then blotted dry under a laminar flow hood (SteriGard Hood, The Baker Company, Inc., Sanford, ME). The outer skin surface was placed toward the inside of a sterile 2.5 cm-diameter bottomless centrifuge tube (Nalgene, CO.) and was kept in place with sterile rubber bands. Excess skin was trimmed and remaining skin was covered with a piece of aluminum foil to prevent contamination and drying. The tube with its bottom covered by skin was placed upright and a 2 ml suspension of  $10^8$ - $10^9$  cfu/ml of GFP-*Campylobacter* was added thereby exposing the outer surface of skin to the bacteria. Attachment was allowed to take place at room temperature (21°C) while shaking on an

orbital shaker at 70 rpm for 1 hr. The cell suspension was then decanted and unattached cells were removed by rinsing 3 times for 1 min each with 2 ml of SDW on an orbital shaker at 100 rpm. The inoculated skin was retrieved by aseptically cutting it from the 2.5 cm-diameter opening of the bottomless centrifuge tube. A negative control was done using the same procedure with 2 ml SDW instead of suspension of *GFP-Campylobacter*. Following exposure, 2.5cm-diameter piece (5 cm<sup>2</sup>) was placed in sterile stomacher bag containing 5ml phosphate buffer saline (PBS) and massaged in a stomacher blender (Stomach<sup>®</sup>80 Biomaster, Seward, England) at high speed for 2 min. Aliquots of the skin wash were serially diluted, spread plated in duplicate on CCA-KM, incubated at 42°C 48 hr to enumerate *GFP-Campylobacter* and also on plate count agar (PCA), at 35°C for 24hr to detect natural microflora on skin. *GFP-Campylobacter* present in wash solution was indicated as loosely and firmly attached cells. Three pieces of inoculated chicken skin and three controls were analyzed in each of replications. Data from chicken skin was not used if the negative control had more than 500 cfu of total aerobic bacteria in 1 cm<sup>2</sup>. Presumptive colonies of *GFP-Campylobacter* were confirmed by observing their motility under a phase contrast microscope (Lobophot, Nikon, Japan) and by a latex agglutination test (INDX-Campy<sup>™</sup>, Integrated Diagnostics, Inc., Baltimore, MD).

**CTC staining:** CTC (Polysciences, Warrington, PA) was diluted to obtain a final concentration of 5 mM with R<sub>2</sub>A broth (Reasoner, 1985). One hundred and fifty microlitres of 5 mM CTC was applied to the *GFP-Campylobacter* inoculated skin as well as the control and incubated in a dark chamber for 30 min at room temperature. CTC-stained skin was then rinsed with 15 ml SDW 3 times (1 min each) on an orbital shaker at



100 rpm. A 1x1-cm specimen was cut at the middle of inoculated chicken skin for visual analysis.

**Visualization of GFP-*Campylobacter* on chicken skin:** Cover well imaging chamber gaskets (Molecular Probes, Eugene, OR) of 2 mm thickness were used to prevent compression of the chicken skin during microscopic observation. A drop of 50% glycerol in PBS was placed into the chamber as a mounting medium. The outer surface of specimen was placed upside down on mounting medium and then securely sealed within the chamber by placing a microscope slide against the gasket surface and pressing gently around the edges of the slide. At least ten microscopic fields were randomly scanned in each specimen. The metabolic active and inactive GFP-*Campylobacter* were determined at different locations and depth under the surface.

**Confocal Scanning Laser Microscope (CSLM):** A TCS NT SP2 Leica Confocal microscope (Leica Microsystems Heidelberg GmbH, Germany) equipped with a 40x (numerical aperture = 1.25, Leica, Germany) and 100x oil immersion objective (numerical aperture = 1.3, Leica, Germany) were used for microscopic observation. An Ar laser (excitation wavelength [ $\lambda$ ] = 488 nm) was used to excite GFP and CTC-formazan. Emitted light was collected through a triple dichroic mirror (TD) 488/568/633. The reflected light (wavelength of 483-495 nm) was assigned as a grey color for chicken skin-reflected light, emitted light (495-540 nm) as green color for GFP image, and 600-670 nm emission as red color for CTC-formazan image. TCS NT software (version

1.6.551; Leica Microsystems) and Adobe Photoshop version 6.0 (Adobe Systems Incorporated, US) were used to process images.

**Validation and Controls:** To determine the ability of CTC to discriminate live from dead cells, GFP *Campylobacter* was killed by overnight exposure to 30% (v/v) alcohol. One ml of cell suspension of untreated cells, killed cells and mixture of killed and untreated cells were stained with 150  $\mu$ l of CTC for 30 min, washed, centrifuged 3 times for 5 min each. Each pellet was resuspended with 1 ml PBS and micrographs obtained using a TCS NT SP2 Leica Confocal microscope (Leica Microsystems Heidelberg GmbH, Germany) with the same collection parameters used to acquire the GFP images and CTC images as mentioned previously.

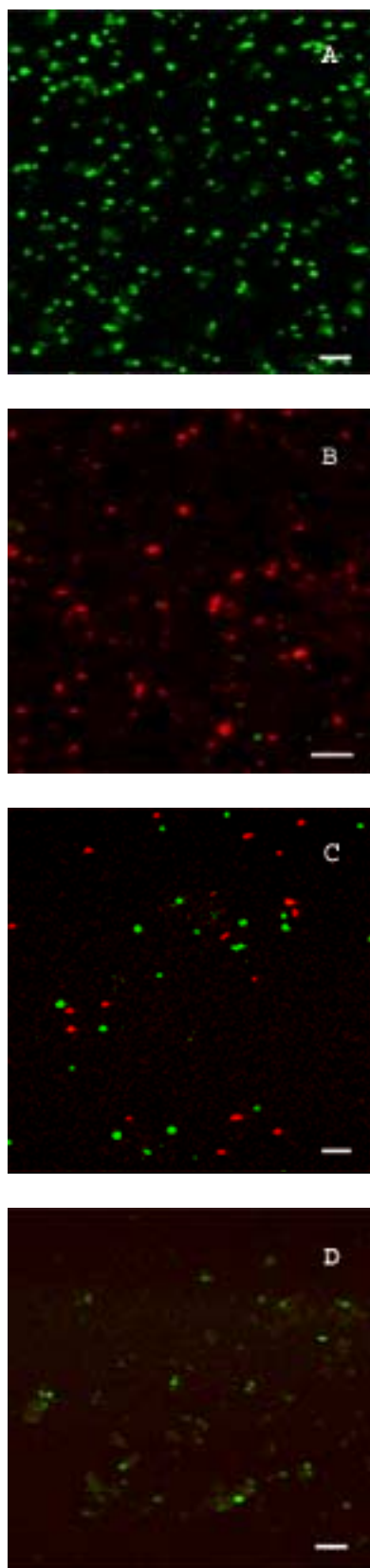
Because of nonselective staining characteristic of CTC and autofluorescence of chicken skin, uninoculated chicken skin, stained with and without CTC were used to calibrate the sensitivity of photomultipliers for detecting GFP-*Campylobacter* and CTC-formazan to reduce noise before scanning.

**Statistical analysis:** All experiments were performed in triplicate. In experiments on enumerating GFP-*Campylobacter* populations from chicken skin and which CSLM was used, each replicate consisted of 3 pieces, making a total of 9 pieces. Data was analyzed with SAS software (SAS Institute, Cary, NC) using PROC ANOVA. Significant differences between means were determined using Least Significant Difference (LSD) test. Significance was determined the 95% confidence level ( $P = 0.05$ ).

## RESULTS AND DISCUSSION

Both live and dead cells in the 48 hour culture of GFP-*Campylobacter* used in this experiment exhibited green fluorescence (Fig. 1.1A). Miller et al. (2000) found that over 90% of cells of this strain exhibited fluorescence after five subcultures. However, fluorescence stability is only a factor when the transformants undergo multiple rounds of cell division causing the proportion of the nonfluorescent subpopulation to increase with successive generations. Therefore, the effect of fluorescence instability is minimal for this attachment experiment.

Intracellular accumulation of CTC-formazan in viable GFP-*Campylobacter* was successfully detected as the bright red fluorescent cells among the green fluorescent dead cells (Fig. 1.1B and 1.1C). Theoretically, viable cells could appear yellow due to green channel overlapping with red channel; and some yellow cells were observed. Most of green fluorescent *C. jejuni* cells were not detected at the same location as the red cells. The green fluorescence of GFP-*Campylobacter* of this experiment was detected at 495-540 nm with 488 nm Ar-Kr laser excitation. Severin et al (1985) showed that CTC-formazan appears to exhibit broad excitation, from less than 400 nm to more than 550 nm with an emission maximum of 630 nm. This means that emission by GFP-*Campylobacter* cells will be intercepted by CTC-formazan when both are in the same cell (either by direct absorption of photons, emitted by GFP-*Campylobacter*, or by Forster energy transfer) (Yu et al., 1995). However, it was also possible that the green fluorescence of GFP-*Campylobacter* was degraded due to cell death, which occurred



GFP-*Campylobacter* when calculating total *Campylobacter* for samples which CTC stain is used.

After 1 h inoculation with 2 ml of  $10^8$ - $10^9$  cfu/ml GFP-*Campylobacter* suspension by the method of Kim et al (1996c) and rinsing,  $10^5$ - $10^6$  cfu of *C. jejuni* remained on 1 cm<sup>2</sup> of chicken breast skin with no statistically difference ( $P>0.05$ ) among three replications (Data not shown).

Green fluorescent *C. jejuni* cells and CTC-formazan in viable *Campylobacter* were clearly visible on the chicken skin by using CSLM. GFP-*Campylobacter* cells remaining on the chicken skin after rinsing were located on rough areas of chicken skin: in crevices, entrapped inside deep channels and feather follicles with water. There were statistical

---

FIG. 1.1. Representative Confocal Scanning Laser Microscope images of live and dead *C. jejuni* transformed with P<sub>c</sub> *gfp* fusion plasmids (GFP *C. jejuni*). (A) Cells of GFP *C. jejuni* without 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining in which green color indicates both dead and live cells. (B) Cells of GFP *C. jejuni* stained with CTC in which red color indicates live cells and green color indicates dead cells. (C) Mixture of live GFP *C. jejuni* and dead GFP *C. jejuni* with CTC staining. (D) Dead GFP *C. jejuni*, which killed by 30% alcohol overnight, stained with CTC. Scale bars represent 5 μm.

when exposed to CTC. Thus, it is necessary to add numbers of CTC stained cells to numbers of differences ( $P < 0.05$ ) among the average of total cells, live cells (able to reduce CTC) and % live cells at different locations after rinsing (Table 1.1). Total cells, live and dead, were located at 0-10 $\mu$ m depth in feather follicles,  $42.4 \pm 23.3$ , more than on the surface,  $27.9 \pm 24.8$ , and at 0-10 $\mu$ m of channel,  $20.1 \pm 9.3$ . There were no significant differences in the mean numbers of live cells or percentage of live cells at depths of 0-10 $\mu$ m in feather follicles, channels or on the surface. However, at depths of 30-50  $\mu$ m below the surface, the average of total cells in feather follicles ( $14.4 \pm 17.3$ ) was greater than in channels at the same depth ( $3.4 \pm 3.4$ ) but less than those found at depths of 0-10 $\mu$ m. When comparing percentage of live cells at different locations, GFP-*Campylobacter* found at 30-50 $\mu$ m deep in feather follicles had a greater chance of survival than those in crevices or within 0-10 $\mu$ m of channel.

Kim et al (1996c) observed *Salmonella* at similar locations on chicken skin and also observed *Salmonella* entrapped with water inside feather follicles at various depths. Live and dead GFP-*Campylobacter* cells were observed deep inside the feather follicles and in deep channels of the skin in this experiment (Fig 1.2). Kim et al (1996a) concluded that the physical structure of chicken skin has a significant role in *Salmonella* attachment which was not influenced by viability or cell surface chemistry.

Water uptake during cleaning and immersion chilling of poultry carcasses may be a significant cause of microbial contamination (Thomas et al, 1987). The microtopography of the tissue is altered by swelling and exposure of deep channels and crevices. The water in these capillary-sized spaces is difficult to remove by conventional cleaning practices and can entrap bacteria providing them an opportunity to penetrate into

the skin pores. Skin swelling may not only trap bacteria already located in channels and crevices rendering them less accessible to physical removal but also provide additional surface skin area for contamination (Thomas and McMeekin, 1980).

GFP-*Campylobacter* were mainly present at 0-10µm from the skin surface. However, the percentage of live cells in this location was low. Fewer GFP-*Campylobacter* cells were found at deeper location but the chance for survival was greater. At the very bottom of most feather follicles and deep channels, we found both active and inactive cells (Fig 1.2), indicating that these sites may provide a suitable microenvironment for *Campylobacter* to survive.

This research demonstrates the feasibility of using a combination of GFP-*Campylobacter* and CTC staining to detect total and viable *Campylobacter* at various sites on chicken skin. Although the formation of fluorescent CTC crystals indicates active reduction by the cell, cells with this ability are not always capable of forming colonies (Federighi et al, 1998). In this study, allowing incubation of samples in CTC for greater than 30 minutes increased nonspecific background fluorescence to the extent that identification of bacterial cells was impaired. CTC-formazan deposition can be observed as small region as 0.2 µm of (Rodriguez et al, 1992). It is possible that the site of CTC-formazan deposition represents areas in cells where electron transport activity is occurring, although this was not proven. Viability counts obtained using CTC staining must be interpreted with caution since all viable cells may not reduce the stain under the experimental conditions, and because nonspecific reduction may make it difficult to discriminate the target bacterial population.

This technique can be used as a method to evaluate the effective of sanitizing agents on viability of *Campylobacter* on chicken skin and detect viability of *Campylobacter* in different storage conditions.

#### ACKNOWLEDGEMENTS

This research was supported by State and Hatch funds allocated to the Georgia Agricultural Experiments stations and by the Royal Thai Government.

## REFERENCES

- Blankenship, L. C. and S. E. Craven. 1982. *Campylobacter jejuni* survival in chicken meat as a function of temperature. Appl. Environ. Microbiol. 44: 88-92.
- Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. M. McGuiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1998. Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. Appl. Env. Microbiol. 64:733-741.
- Cappelier, J. M., B. Lazaro, et al. 1997. Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable *Campylobacter jejuni* cells. Vet. Res. 28: 547-555.
- Centers for Disease Control and Prevention. 2002. Preliminary FoodNet Data on the Incidence of Foodborne Illnesses - Selected Sites, United States, 2001. Morbid. Mortal. Weekly Rep. 51(15);325-9
- Federighi, M., J. L. Tholozan, J. M. Cappelier, J. P. Tissier, and J. L. Jouve. 1998. Evidence of non-coccoid viable but non-culturable *Campylobacter jejuni* cells in microcosm water by direct viable count, CTC-DAPI double staining, and scanning electron microscopy. Food Microbiol. 15: 539-550.
- Kim, J., S. J. knabel and S. Doores. 1993a. Penetration of *Salmonella typhimurium* into turkey skin. J. Food Prot. 56:292-296.
- Kim, J., M. F. Slavik, C. L. Griffis, and J. T. Walker. 1993b. Attachment of *Salmonella typhimurium* to skins of chicken scalded at various temperatures. 56:661-665.
- Kim, J. W. and S. Doores. 1993c. Influence of three defeathering systems on microtopography of turkey skin and adhesion of *Salmonella typhimurium*. Food Prot. 5:286-291.
- Kim, K. Y., J. F. Frank, and S. E. Craven. 1996a. Attachment of *Salmonella typhimurium* to poultry skin as related to cell viability. J. food Sci. 61:439-441, 448.
- Kim, K. Y., J. F. Frank, and S. E. Craven. 1996b. Attachment of *Salmonella* on modified poultry skin surface. J. food Sci. 61:442-443, 448.
- Kim, K. Y., J. F. Frank, and S. E. Craven. 1996c. Three-dimentional visualization of *Salmonella* attachment to poultry skin using confocal scanning laser microscopy. Letters in Applied Microbiology 22: 280-282.



- Lee, A., S. C. Smith, and P. J. Coloe. 1998. Survival and growth of *Campylobacter jejuni* after artificial inoculation onto chicken skin as a function of temperature and packaging conditions. *J. Food Prot.* 61(12): 1609-1614.
- Miller, W. G., A. H. Bates, S. T. Horn, M. T. Brandl, M. R. Wachtel, and R. E. Mandrell. 2000. Detection on surfaces and in caco-2 cells of *Campylobacter jejuni* cells transformed with new *gfp*, *yfp*, and *cfp* marker plasmids. *Appl. Environ. Microbiol.* 66(12): 5426-5436.
- Reasoner, D. J. and E. E. Geldreich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* 49:1-7.
- Rodriguez, G., Phipps, D., Ishiguro, K., and Ridgway, H. F. 1992. Use of a Fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* 58(16):1801-1808.
- Severin, E., J. Stellmach, and H. Nachtigal. 1985. Fluorimetric assay of redox activity in cells. *Anal. Chim. Acta.* 170:341-346.
- Simmons, N. A. and F. J. Gibbs. 1979. *Campylobacter* subsp. in oven-ready poultry. *J. Infect.* 1: 159-162.
- Stellmach, J. 1984. Fluorescent redox dyes. I. Production of fluorescent formazan by unstimulated and phorbol ester or digitonin-stimulated Ehrlich ascites tumor cells. *Histochem. J.* 80: 137-143.
- Stern, N. J. 1992a. Reservoirs for *Campylobacter jejuni* and approaches for intervention in poultry. *In Campylobacter jejuni: current status and future trends.* I. Nachamkin, M. J. Blaser and L. S. Tompkins (eds). American Society for Microbiology. Washington, D.C. 49-60.
- Stern, N. J., B. Wojton, and K. Kwiatek 1992b. A differential selective medium and dry ice generated atmosphere for recovery of *Campylobacter jejuni*. *J. Food Prot.* 55:514-517.
- Tholozan, J. L., J. M. Cappellet, J. P. Tissier, G. Delattre, and M. Federighi. 1999. physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. *Appl. Environ. Microbiol.* 65(3): 1110-1116.
- Thomas, C. J. and T. A. McMeekin. 1980. Contamination of broiler carcass skin during commercial processing procedure: an electron microscopic study. *Appl. Environ. Microbiol.* 40:133-144.
- Thomas, C. J., T. A. McMeekin and J. T. Patterson. 1987. Prevention of microbial contamination in the poultry processing plant. *In Elimination of pathogenic organisms from meat and poultry.* F. J. M. Smulders (ed) Elsevier, New York

Yu, W., W. K. Dodds, M. K. Banks, J. skalsky, and E. A. Strauss. 1995. Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes. *Appl. Environ. Microbiol.* 61(9)3367-3372.

**Table 1.1:** Number of live, total GFP-*Campylobacter* cells, and % live cells at different locations on chicken skin as determined by direct microscopic observation.

Type (cells)	Number of cells (cells/field) <sup>a</sup>					
	Location on skin (depth of image)					
	Surface (8.4±2.4µm) <sup>b</sup>	Crevice (16.9±2.7µm) <sup>b</sup>	Channel (49.4±16.7µm) <sup>b</sup>		Feather follicle (42.4±23.3µm) <sup>b</sup>	
			0-10µm <sup>c</sup>	30-50µm	0-10µm <sup>c</sup>	30-50µm
Live <sup>e</sup>	4.3± 5.8AB <sup>d</sup>	4.2± 4.1AB	4.1± 3.1AB	0.3± 0.6C	5.7± 5.6A	2.3± 2.8BC
Total	27.9±24.6B <sup>d</sup>	16.0± 6.7C	20.1± 9.3BC	3.4± 3.4D	42.4±23.3A	14.4±17.3C
% Live <sup>f</sup>	15.89± 20.08B	26.66±21.99AB	25.09±26.94AB	14.70±32.36B	14.49±18.23B	35.16±38.23A

<sup>a</sup> Values were the means and standard deviation of three replications. Number of GFP-*Campylobacter* cells was determined per microscopic field ( $6.25 \times 10^4 \mu\text{m}^2$ ) within different locations.

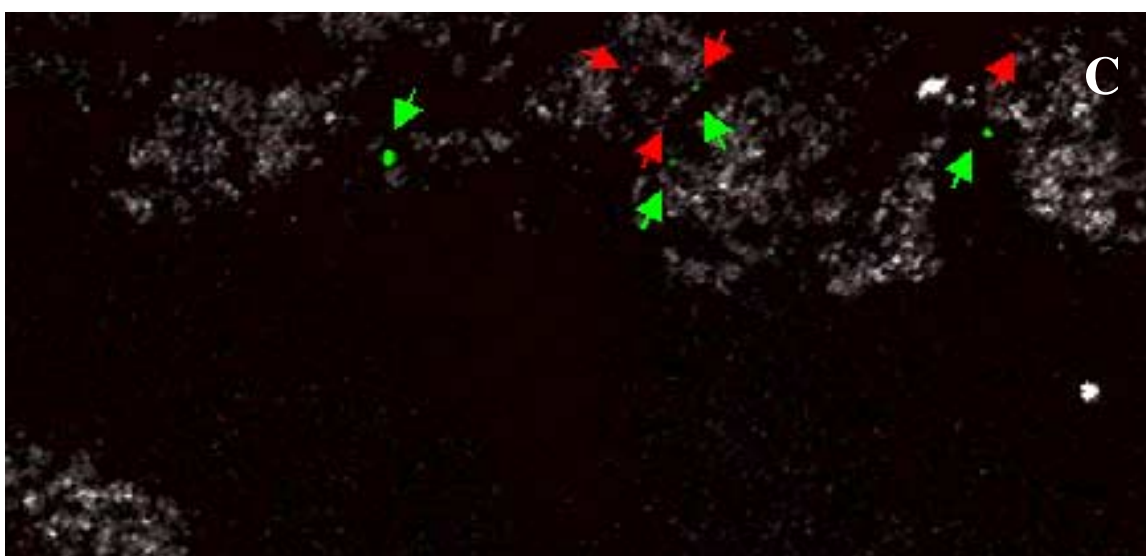
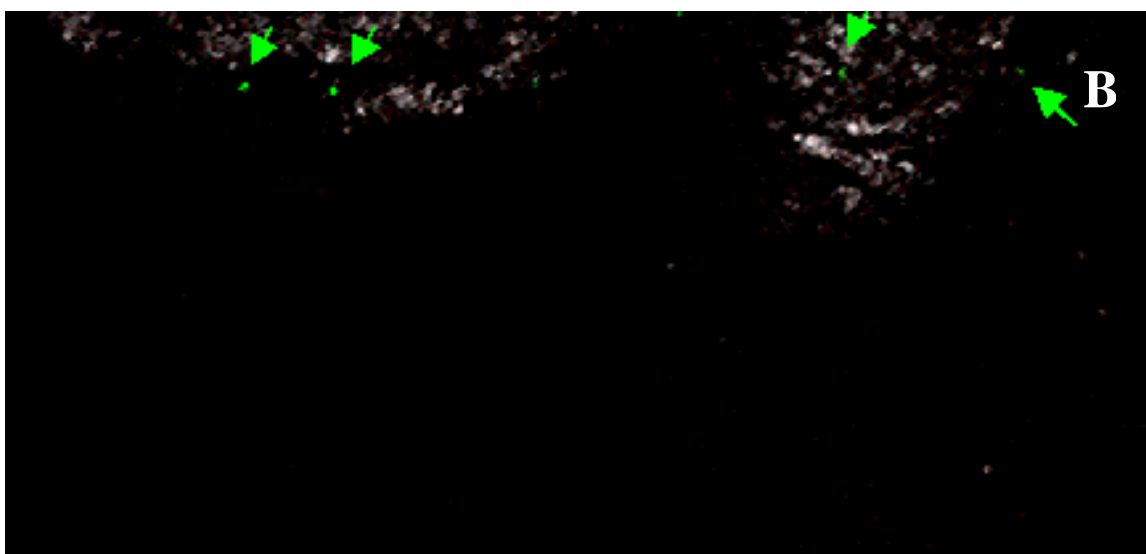
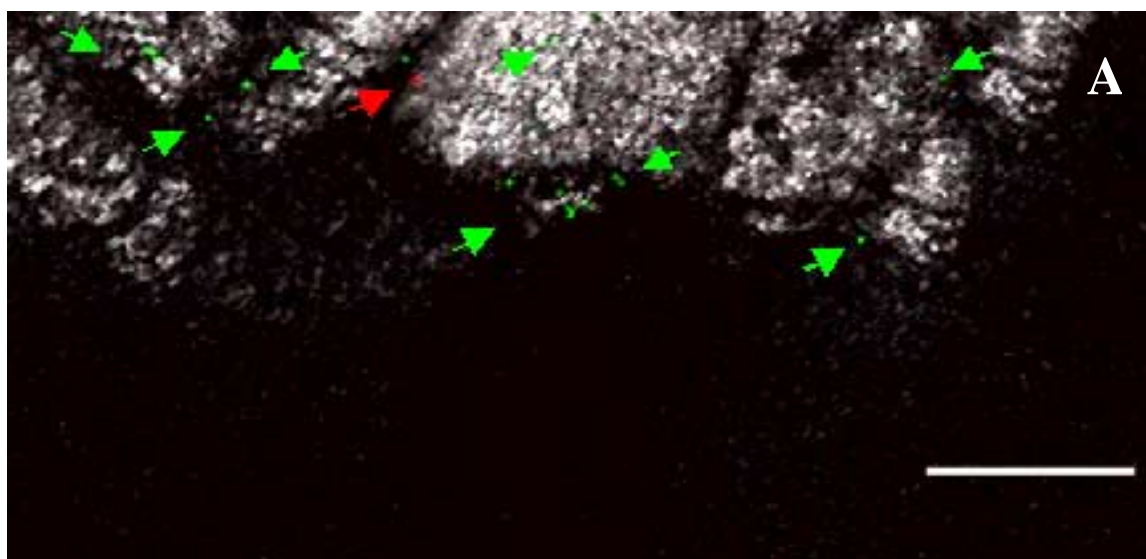
<sup>b</sup> Values were the means and standard deviation of distance from the upper most position of specimen which firstly seen under CSLM till the end of different locations from three replications. <sup>c</sup> Distance from the channel surface or feather follicle surface.

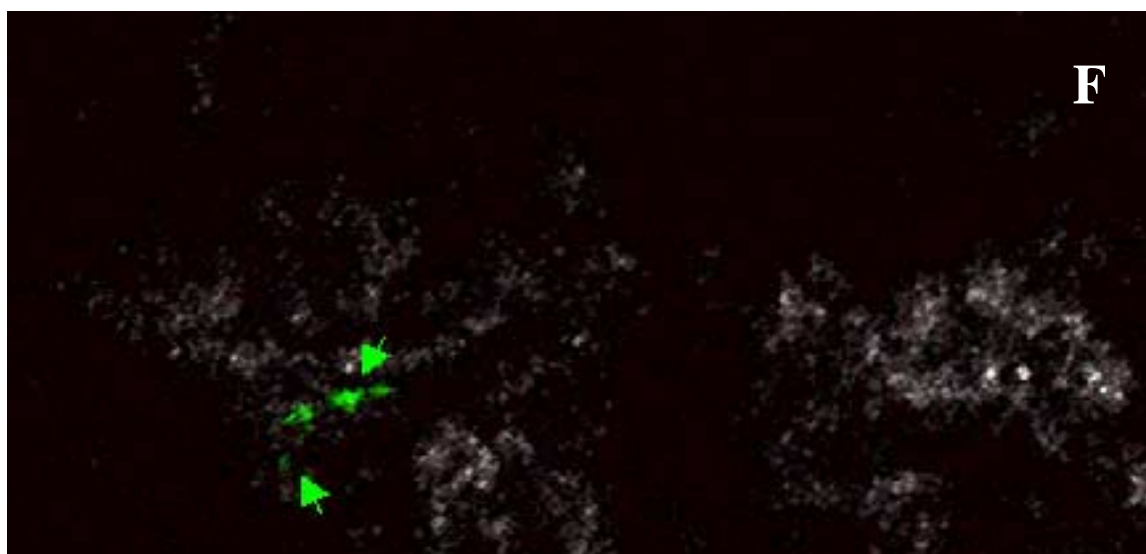
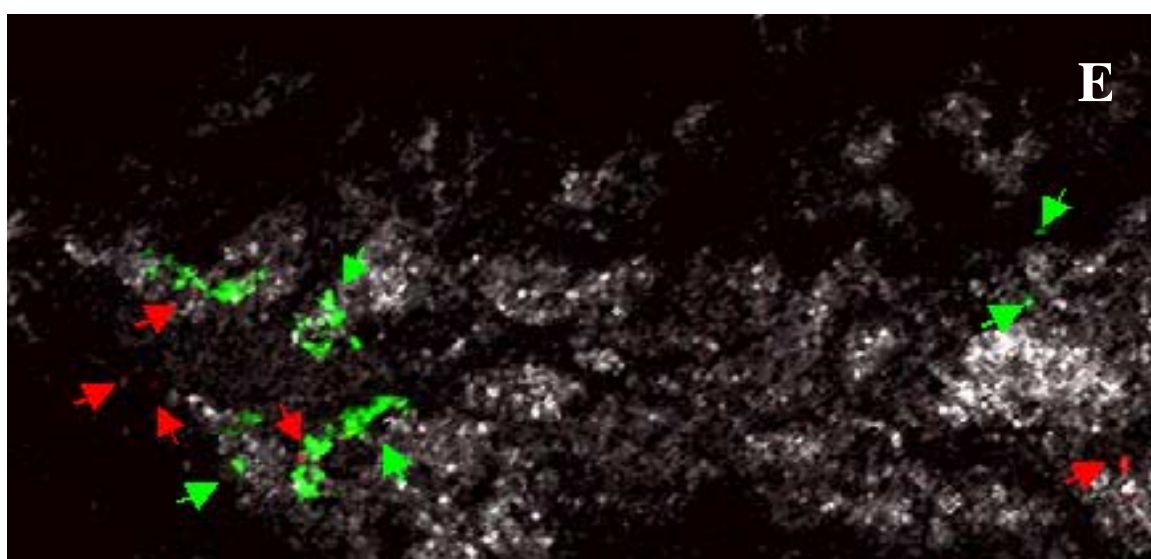
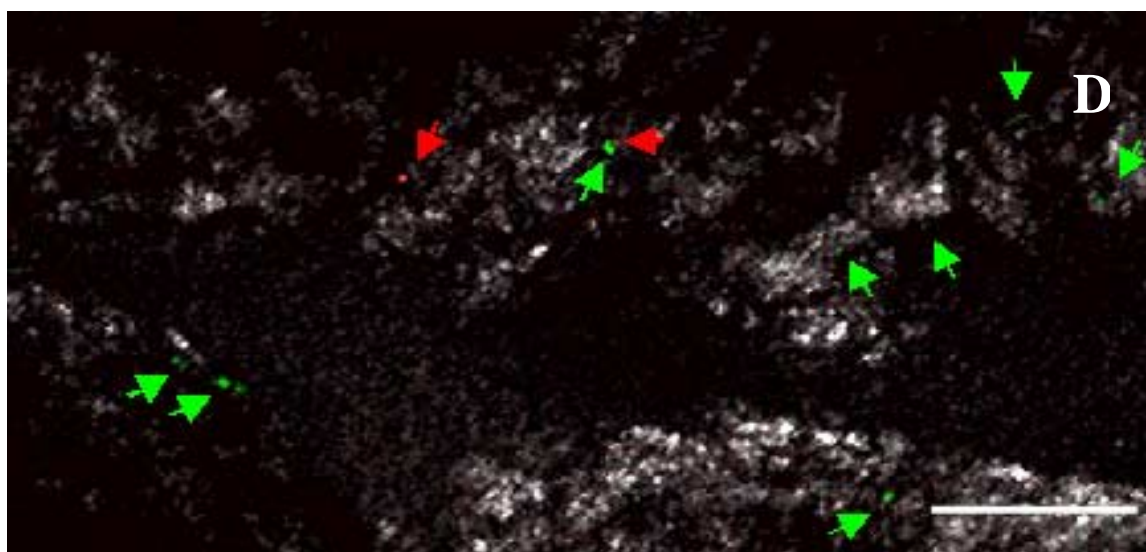
<sup>d</sup> Different letters in the same row indicate significant differences ( $P < 0.05$ ) using LSD test..

<sup>e</sup> Live cells represented cells which contain red crystal of CTC-formazan.

<sup>f</sup> % Live cells = (numbers of live cells)/(number of total cells) x 100. Values were the means of three replications and standard deviation.

Figure 1.2 (A-F). Representative z section scan of GFP-*Campylobacter* trapped in feather follicles, 29.6  $\mu\text{m}$  deep. Successive images (A-F) started from the surface, 0  $\mu\text{m}$ , (image A) to 29.6  $\mu\text{m}$  below the skin surface (image F) with 5.9  $\mu\text{m}$  apart. Green arrows indicate dead cells, green cells, and red arrows indicate live cells, red cells. Scale bar represents 20  $\mu\text{m}$ .





## CHAPTER 2

### DIRECT MICROSCOPIC OBSERVATION OF *CAMPYLOBACTER JEJUNI* SURVIVAL ON CHICKEN SKIN AS AFFECTED BY TEMPERATURE<sup>1</sup>

---

<sup>1</sup>Chantarapanont, W., M. Berrang, and J. F. Frank. 2002. To be submitted to Journal of Food Protection

## ABSTRACT

*Campylobacter jejuni* are commensal in the intestinal tract of a wide variety of animals especially poultry. The chicken intestinal tract, ceca and colon, which contain high levels of *C. jejuni* may leak during processing especially during defeathering and evisceration, resulting in adding *C. jejuni* contamination to the skin of the carcass. The objective of this research is to observe survival of GFP-*Campylobacter jejuni* cells at different depths on chicken skin stored at different temperatures. The method we employed used confocal laser scanning laser microscopy (CSLM) visualization of *Campylobacter jejuni* transformed with P<sub>cgfp</sub> plasmid (GFP-*Campylobacter*) exposed to 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). The population of *C. jejuni* on chicken skin decreased during storage at 25°C for 24hr. Storage at 25°C did not affect viability of *Campylobacter jejuni* located beneath the chicken skin, 20-30µm. *C. jejuni* on chicken skin stored at 4°C slightly increased from 0hr to 72hr and the viable cells of GFP-*C. jejuni* increased at both surface and 20-30µm of chicken skin. Live and dead cells are initially retained with water on the skin and penetrate into the skin with time. Chicken skin provides a suitable environment for *Campylobacter* to survive and grow at 25°C and 4°C, respectively.



Although several bacterial pathogens have been associated with poultry-borne human illnesses, *Campylobacter jejuni* is the major concern. Campylobacters from a variety of sources on the farm colonize poultry are carried to processing plants. Even in sanitary modern processing plants, campylobacters are present on poultry throughout processing operations. Scalding, defeathering, evisceration and giblet operations are the major points of transfer of microorganisms (Bryan and Doyle, 1995). The chicken intestinal tract, ceca and colon, which contain high levels of *C. jejuni* may leak during processing especially during defeathering, and result in adding *C. jejuni* contamination to the skin of the carcass (Berrang, 2000).

The outer skin of chicken is a major site of carcass contamination (McMeekin et al., 1984). *C. jejuni* is retained in a liquid film on the skin and becomes entrapped in skin ridges and crevices. The process of retention begins on live birds and is enhanced during scalding (Thomas and McMeekin, 1980). Scalding opens feather follicles to aid feather removal, and the follicles remain open throughout processing until chilling when they close thereby retaining microorganisms. Due to the microaerophilic requirements of *C. jejuni*, chicken skin can provide a microenvironment for *C. jejuni* to survive. However, the numbers of *C. jejuni* on carcass surfaces have been increased and decreased at different stages of processing (Berrang et al., 2000).

Lee et al (1998) found that *C. jejuni* could persist at high numbers or grow rapidly in chicken skin stored at 4°C and at ambient room temperature in controlled atmosphere packaging. It is possible that growth of food-spoilage microorganisms which increased 10- to 100-fold during storage influence the behavior of *C. jejuni*. However, this

relationship was not determined by Lee et al (1998). Blankenship and Craven (1982) reported that *C. jejuni* survived well with spoilage microflora including both *Pseudomonas* sp. predominant in air packaged and *Lactobacillus* sp. in carbon dioxide packaged products stored at low temperature.

The objective of this research was to observe survival of GFP-*Campylobacter jejuni* cells at different depths on chicken skin stored at different temperatures. This information will help explain how *C. jejuni* survives on chicken skin, and may be useful in the development of technologies for eliminating *C. jejuni* from the skin. CTC, 5-cyano-2,3-ditolyl tetrazolium chloride, is a monotetrazolium redox dye which produces a fluorescent formazan (CTF), red crystal, when it is chemically or biologically reduced. We used this dye to observe live GFP-*Campylobacter jejuni* and natural microflora on chicken skin. CTC staining was previously demonstrated to provide viable counts similar to the cell elongation method (direct viable count, DVC) method for *C. jejuni* (Cappelier et al., 1997).

## MATERIALS AND METHODS

**Bacterial strain and plasmid:** GFP *Campylobacter jejuni* RM 1221 (Pwm1007) was provided by W. G. Miller USDA Agricultural Research Service, Albany, CA. The plasmid encoding GFP (Pwm1007; Km<sup>r</sup>; Pmw10 $\Delta$ lacZ $\Omega$ [(T1)<sub>4</sub>-P<sub>c</sub>-gfp-T1] was mobilized into *C. jejuni* strain RM 1221 from a chicken carcass isolate (Miller et al., 2000). Transformants were selected by plating on Cefex-campy agar (CCA) (Stern et al., 1992) amended with 200  $\mu$ g of kanamycin per ml (CCA-KM) and grown at 42°C for 48 hr in

sealed bag with a mixture of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub> (BOC Gases, Chattanooga, TN). Colonies on CCA-KM were restreaked on fresh plates, and expression of GFP-*Campylobacter* was confirmed by observation of bacterial smears with a Confocal Scanning Laser Microscope (CSLM) as describe in following section. Kanamycin-resistant clones, which exhibited green fluorescent by microscopic observation were maintained at -70°C in 0.1% phosphate buffered saline (PBS) with sterile 20% glycerol.

**Culture Preparation:** Stock cultures of GFP-*Campylobacter* were resuscitated by streaking on CCA-KM and incubated at 42°C for 48 hr in a sealed bag containing of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>. Cultures were transferred at least twice before using. For the attachment assay, a loopful of isolated colonies of GFP-*Campylobacter* on CCA-KM agar was suspended in sterile deionized water (SDW). The optical density (O.D.) of the cell suspension was adjusted to 0.5 – 0.6 using a spectrophotometer (Spectronic 21D; Milton Roy Co., Rochester, NY) at 540 nm to yield 10<sup>8</sup> – 10<sup>9</sup> cells/ml.

**Chicken skin:** Freshly processed broiler carcasses were randomly collected immediately upon exiting the chill tank in a commercial processing plant. Carcasses were individually bagged and stored in ice no more than 30 minutes before removal of breast skins. Breast skin (6 x 6 cm section) was removed from the same location on breasts of broiler carcasses for each assay using sterile scalpel and forceps. Chicken skin was aseptically stored at -20°C, no more than one week before using.

**Attachment assay:** Attachment was determined as described by Kim et al (1996). Frozen chicken skins were thawed by rinsing 3 times for 3 min each with 15 ml of 4°C SDW on an orbital shaker model No. 3520 (Lab Line Instrument Inc, IL) at 100 rpm. Skins were then blotted dry under a laminar flow hood (SteriGard Hood, The Baker Company, Inc., Sanford, ME). The outer skin surface was placed toward the inside of a sterile 2.5 cm-diameter centrifuge tube (Nalgene, CO.) with the bottom removed, and was kept in place with sterile rubber bands. Excess skin was trimmed and remaining skin was covered with a piece of aluminum foil to prevent contamination and drying. The tube with its bottom covered by skin was placed upright and a 3 ml suspension of  $10^8$ - $10^9$  cfu/ml of GFP-*Campylobacter* was added thereby exposing the outer surface of skin (at the bottom of the tube) to the bacteria. Attachment was allowed to take place at room temperature (21°C) while shaking on an orbital shaker at 70 rpm for 1 hr. The cell suspension was then decanted and unattached cells were removed by rinsing 3 times for 1 min each with 2 ml of SDW on an orbital shaker at 100 rpm. The negative control used the same procedure with 3 ml SDW instead of the suspension of GFP-*Campylobacter*.

**Microbiological Analysis:** After rinsing, the tubes with chicken skin were inverted and stored at 25°C or 4°C. Chicken skins stored at 25°C were sampled at 0, 8, 16, and 24 hr and those at 4°C were sampled at 0, 24, 48, 72, and 96hr. The inoculated skin was retrieved by aseptically cutting it from the 2.5 cm-diameter opening of the bottomless centrifuge tube. Then 2.5cm-diameter piece ( $5\text{ cm}^2$ ) of inoculated chicken skin was placed in sterile stomacher bag containing 5 ml of an enrichment broth medium, BR Tween, which contain 28 g/litre brucella broth (Acumedia Manufactures, inc.,

Baltimore, MD), 1g/litre Tween 80<sup>TM</sup> (Sigma), 33 mg/litre cefoperazone, FBP (as described previously) and Campylobacter selective supplement (Bolton's) (Dalynn Biologicals, Calgary, Alberta, Canada) used as recommended by the manufacturer and massaged in a stomacher blender (Stomach<sup>®</sup> 80 Biomaster, Seward, England) at high speed for 2 min. Aliquots of the skin wash were serially diluted, spread plated in duplicate on CCA-KM, and incubated at 42°C 48 hr to enumerate GFP-*Campylobacter*. Five ml of phosphate buffer saline (PBS) were placed in sterile stomach bag with the uninoculated control and the natural microflora on skin was enumerated by using plate count agar (PCA) incubated at 35°C for 24hr. Three pieces of inoculated chicken skin and three controls were analyzed at each sampling time in each replication. Data from chicken skin was not used if the uninoculated control initially had more than 500 cfu of total aerobic bacteria in 1 cm<sup>2</sup>. Presumptive colonies of GFP-*Campylobacter* were confirmed by observing their motility under a phase contrast microscope (Lobophot, Nikon, Japan) and by a latex agglutination test (INDX-Campy<sup>TM</sup>, Integrated Diagnostics, Inc., Baltimore, MD).

**CTC staining:** CTC, 5-cyano-2,3-ditolyl tetrazolium chloride, (Polysciences, Warrington, PA) was diluted to obtain a final concentration of 5 Mm with R<sub>2</sub>A broth (Reasoner and Geldreich, 1985). One hundred and fifty microlitres of 5 Mm CTC was applied to the skin sample and incubated in a dark chamber for 30 min at room temperature. CTC-stained skin was then rinsed with 15 ml SDW 3 times (1 min each) on an orbital shaker at 100 rpm. A 1x1-cm specimen was cut from the middle of skin sample for microscopic analysis.

**Visualization of GFP-*Campylobacter* on chicken skin:** Cover well imaging chamber gaskets (Molecular Probes, Eugene, OR) of 2 mm thickness were used to prevent compression of the chicken skin during microscopic observation. A drop of 50% glycerol in PBS was placed into the chamber as a mounting medium. The outer surface of specimen was placed upside down on mounting medium and then securely sealed within the chamber by placing a microscope slide against the gasket surface and pressing gently around the edges of the slide. At least ten microscopic fields were randomly scanned for each specimen. The metabolic active and inactive GFP-*Campylobacter* was determined at different depths from the surface.

**Confocal Scanning Laser Microscope (CSLM):** A TCS NT SP2 Leica Confocal microscope (Leica Microsystems Heidelberg GmbH, Germany) equipped with a 40x (numerical aperture = 1.25, Leica, Germany) and 100x oil immersion objective (numerical aperture = 1.3, Leica, Germany) were used for microscopic observation. An Ar laser (excitation wavelength [ $\lambda$ ] = 488 nm) was used to excite GFP and CTC-formazan. Emitted light was collected through a triple dichroic mirror (TD) 488/568/633. The reflected light (wavelength of 483-495 nm) was assigned as a grey color for chicken skin-reflected light, emitted light (495-540 nm) as green color for GFP image, and 600-670 nm emission as red color for CTC-formazan image. TCS NT software (version 1.6.551; Leica Microsystems) and Adobe Photoshop version 6.0 (Adobe Systems Incorporated, US) were used to process images. Because of nonselective staining characteristic of CTC and autofluorescence of chicken skin, uninoculated chicken skin,

stained with and without CTC were used to calibrate the sensitivity of photomultipliers for detecting GFP-*Campylobacter* and CTC-formazan to reduce noise before scanning. ImageJ 1.27z (National Institutes of Health, USA) was used for image analysis.

**Statistical analysis:** All experiments were replicated in triplicate. In experiments in which GFP-*Campylobacter* was enumerated on chicken skin, each sampling time consisted of 3 pieces of inoculated skin, making a total of 9 pieces for each sampling time and for CSLM observation, 2 pieces of inoculated skin were used for CTC-stained for each sampling time, making a total of 6 pieces for each sampling time. Controls were done the same manner. Data was analyzed with SAS software (SAS Institute, Cary, NC) using PROC ANOVA. Significant differences between means were determined using Least Significant Difference (LSD) test. Significance was determined the 95% confidence level ( $P = 0.05$ ).

## RESULTS AND DISCUSSION

The average number of total aerobic bacteria enumerated from uninoculated chicken skin, control, at 0hr was a  $2.53 \log \text{ cfu/cm}^2$  as determined by plating on PCA and  $3.11 \log \text{ cfu/cm}^2$  as determined by direct cell count of CTC stained samples (Table 2.1). Only chicken skin with no more than 500 cfu ( $2.69 \log$ ) / $\text{cm}^2$  of total aerobic bacteria at 0hr was used in this experiment. CTC stained cells were observed initially on the surface (0-10 $\mu\text{m}$  depth) but after being stored at 25°C for 8hr and 4°C for 48hr, they were observed at greater depth (20-30 $\mu\text{m}$ ) although at low numbers. Plate counts indicated

that total aerobic bacteria on chicken skin was as high as  $7.29 \log \text{ cfu/cm}^2$  after storage at  $25^\circ\text{C}$  for 16hr but viable cells in these samples were too numerous to count via the direct microscopic method. The number of total aerobic bacteria was increased by 1 log  $\text{cfu/cm}^2$  with PCA and by  $1.5 \log \text{ cfu/cm}^2$  with DMC after stored at  $4^\circ\text{C}$  for 96 hours. Most of these bacteria were located at the surface.

The average number of GFP-*Campylobacter* on chicken skin after inoculation (0 hr) was  $6.01 \log \text{ cfu/cm}^2$  as determined by plating on CCA-KM and  $5.79 \log \text{ cfu/cm}^2$  of viable *Campylobacter* (after subtracting the average number of total aerobic bacteria on the uninoculated control observed by DMC in Table 2.1). The average number of live cells determined by DMC tended to be lower than that enumerated on CCA-KM possibly because some labeled cells were missed due to low amounts of CTF deposition (Rodriguez et al., 1992). It is also possible that injured cells can form colonies but not be able to reduce CTC. Because the natural microflora on chicken skin also reduced CTC, data on number of live GFP-*Campylobacter* and total number of GFP-*Campylobacter* observed by direct microscopic observation could be obtained only up to 8 hr for samples were stored at  $25^\circ\text{C}$  and up to 72 hr at  $4^\circ\text{C}$ , after which time the natural microflora overgrew the campylobacter.

The average cfu's of GFP-*Campylobacter* on chicken skin (CCA-KM) decreased  $1 \log \text{ cfu/cm}^2$  at a constant rate after 24 hr storage at  $25^\circ\text{C}$  (Fig 2.1 A- $25^\circ\text{C}$ ). The total number of active GFP-*Campylobacter* on chicken skin and at the surface ( $25^\circ\text{C}$ ) as determined by direct viable count decreased with the same trend as the data obtained using the plate count method; however, the number of active GFP-*Campylobacter* at 20-30  $\mu\text{m}$  were constant. This indicated that there was no effect of  $25^\circ\text{C}$  storage on viability



of *Campylobacter jejuni* located 20-30  $\mu\text{m}$  beneath the chicken skin. The population of *C. jejuni* on chicken skin stored at 4°C slightly increased from 0hr to 72hr and direct microscopic count indicated that viable cells of GFP-*C. jejuni* increased at location on the surface and at 20-30 $\mu\text{m}$  depth. Lee et al (1998) reported that *C. jejuni* was able to persist at high numbers or replicate quickly under normal packaging on packaged chicken skin stored at 4°C for 7 days and ambient room temperature for 3 days.

Kim et al (1996) found that viability and cell surface factors do not play significant roles in *Salmonella* attachment to poultry skin, which is primarily influenced by the skin physical structure. Our data demonstrated that GFP-*Campylobacter* cells penetrate into skin over time regardless of viability. However, the majority of live or inactive cells remained present on the surface at both 4°C and 25°C storage. There was an increase of GFP-*Campylobacter* at a depth of 20-30 $\mu\text{m}$  when skin was stored at 25°C for 24hr (Fig 2C, 25°C); however, an association of viability could not be determined at this time. Data from this study support the view that live and dead campylobacters are initially retained with water on the skin and penetrate into the skin over time.

*Campylobacter* that penetrate in crevices and follicles would be difficult to remove from skin because of capillary action, or irreversible attachment to the skin tissue.

Decontamination procedures during processing are required to reduce the population of *C. jejuni* on chicken skin due to the spontaneous penetration of *Campylobacter* during storage. The effectiveness of sanitizers in inactivation *C. jejuni* located under the surface of chicken skin is needed to be evaluated.

Attached bacteria are able to spread in the kitchen by easily transferred to the drip water and later to hands and utensils or other foodstuffs if lack of appropriate food

hygiene processes (Notermans et al., 1975). It is important to separate cooked food from raw foods to avoid cross-contamination as it can survive or rapidly replicate in room temperature and under refrigeration at 4°C (Lee et al., 1998). It has been suggested to cook chicken thoroughly to ensure that *Campylobacter* and all pathogenic bacteria are destroyed.

### **ACKNOWLEDGEMENTS**

This research was supported by State and Hatch funds allocated to the Georgia Agricultural Experiments stations and the Royal Thai Government.

## REFERENCES

- Berrang, M. E., R. J. Buhr, and J. A. Cason. 2000. *Campylobacter* recovery from external and internal organs of commercial broiler carcass prior to scalding. *Poult. Sci.* 79: 286-290.
- 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: 43-47.
- Berrang, M. E., R. J. Buhr, J. A. Cason, and J. A. Dickens. 2001. Broiler carcass contamination with *Campylobacter* from feces during defeathering. *J. Food Prot.* 64(12): 2063-2066.
- Blankenship, L. C. and S. E. Craven. 1982. *Campylobacter jejuni* survival in chicken meat as a function of temperature. *Appl. Environ. Microbiol.* 44: 88-92.
- Bryan, F. L. and M. P. Doyle. 1995. Health risks and consequences of *Salmonella* and *Campylobacter jejuni* in raw poultry. *J. Food Prot.* 58(3): 326-344.
- Cappelier, J. M., B. Lazaro, A. Rossero, A. Fernandez-Astorga, and M. Federighi. 1997. Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable *Campylobacter jejuni* cells. *Vet. Res.* 28: 547-555.
- Kim, K. Y., H. S. Lillard, J. F. Frank, and S. E. Craven. 1996. Attachment of *Salmonella typhimurium* to poultry skin as related to cell viability. *J. Food Sci.* 61(2): 439-441.
- Lee, A., S. C. Smith, et al. 1998. Survival and growth of *Campylobacter jejuni* after artificial inoculation onto chicken skin as a function of temperature and packaging conditions. *J. Food Prot.* 61(12): 1609-1614.
- McMeekin, T. A., C. J. Thomas, and P. I. Pennington. 1984. Contamination and decontamination of poultry carcass neck tissue. *J. Food Safety.* 6: 79-88.
- Miller, W. G., A. H. Bates, S. T. Horn, M. T. Brandl, M. R. Wachtel, and R. E. Mandrell. 2000. Detection on surfaces and in caco-2 cells of *Campylobacter jejuni* cells transformed with new *gfp*, *yfp*, and *cfp* marker plasmids. *Appl. Environ. Microbiol.* 66(12): 5426-5436.
- Notermans, S. and E. H. Kampelmacher. 1975. Studies of different sampling methods for the determination of bacterial counts from frozen broilers. *J. Appl. Bacteriol.* 39: 125-131.
- Reasoner, D. J. and E. E. Geldrich. 1985. A new medium for the enumeration and

subculture of bacteria from potable water. Appl. Environ. Microbiol. 49: 1-7.

Rodriguez, G. G., D. Phipps, K. Ishiguro, and H. F. Ridgway. 1992. Use of a Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bacteria. Appl. Environ. Microbiol. : 1801-1808.

Stern, N. J., B. Wojton, and K. Kawiatek. 1992. A differential selective medium and dry ice generated atmosphere for recovery of *Campylobacter jejuni*. J. Food Prot. 55: 514-517.

Thomas, C. J. and T. A. McMeekin. 1980. Contamination of broiler carcass skin during commercial processing procedures: an electron microscopic study. Appl. Environ. Microbiol. 40: 133-144.

**Table 2.1:** Microflora on uninoculated chicken skin stored at 4°C and 25°C enumerated by plating on Plate Count Agar (PCA) and by direct microscopic count (DMC) using CTC staining.

Temperature	Time (Hour)	Microbial Population			
		PCA (log cfu/cm <sup>2</sup> )	DMC <sup>a</sup>		
			0-10µm	20-30µm	Total <sup>b</sup>
	0	2.53±0.26B <sup>c</sup>	3.11±0.09A	0±0C	3.11±0.09A
25°C	8	4.67±0.28A	4.50±0.70AB	3.44±0.62B	4.54±0.61AB
	16	7.29±0.51	> 5.50 <sup>d</sup>	> 5.50	> 5.50
	24	8.74±0.94	> 5.50	> 5.50	> 5.50
4°C	24	3.01±0.14A	3.99±0.77A	0±0B	3.99±0.77A
	48	3.28±0.26B	4.65±0.55A	3.66±0.57B	4.67±0.54A
	72	3.36±0.15B	4.69±0.16A	4.09±0.59B	4.79±0.18A
	84	3.71±0.30C	4.91±0.14A	4.47±0.33B	5.05±0.18A
	96	4.14±0.04B	5.31±0.55A	4.69±0.44AB	5.41±0.52A

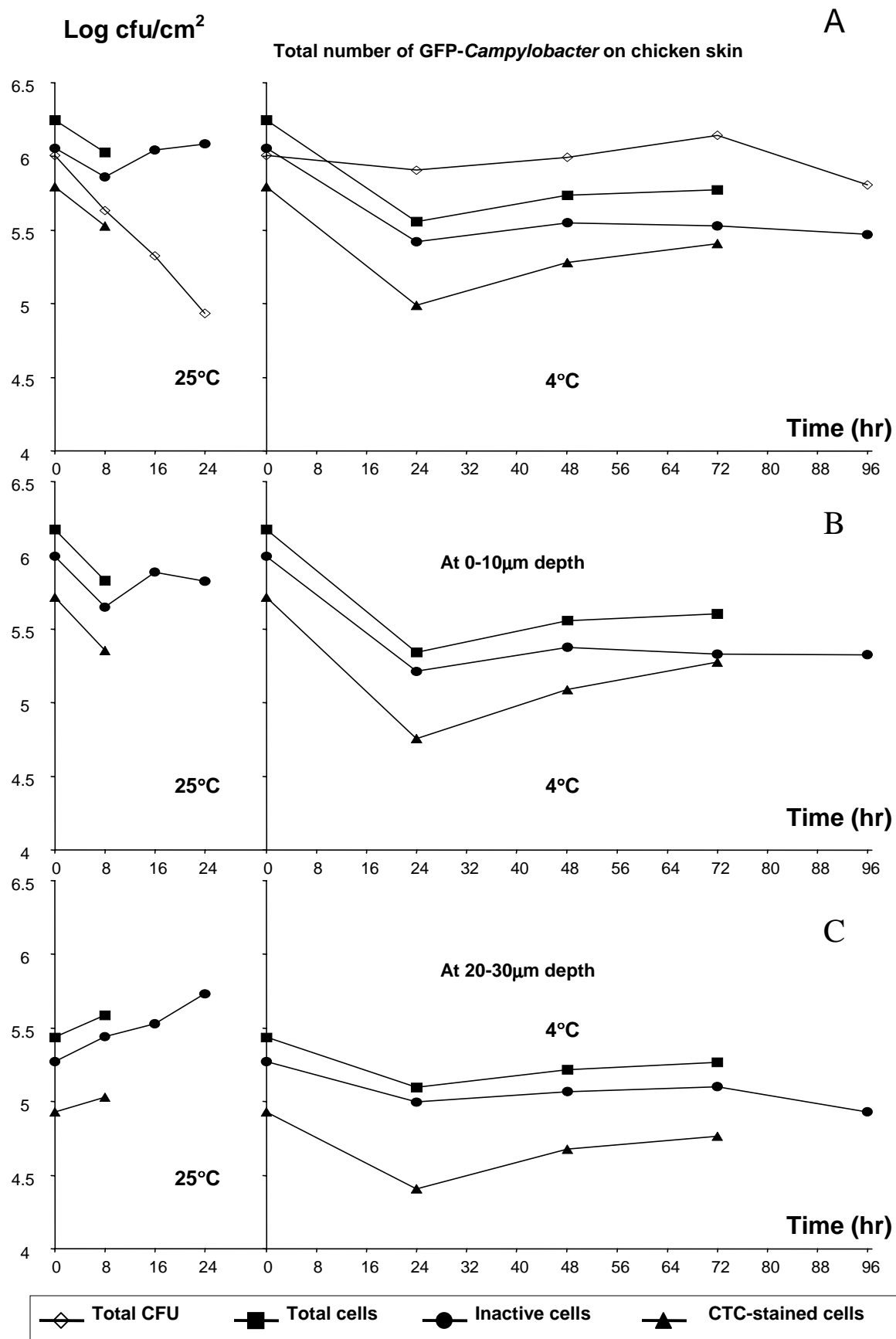
<sup>a</sup>CTC stained cells/cm<sup>2</sup>

<sup>b</sup>Total was calculated by adding from the 0-10 and 20-30µm data.

<sup>c</sup>Values were the means and standard deviation of log cfu/cm<sup>2</sup> of microbial population on control chicken skin. Different letters in the same row indicate significant differences ( $P<0.05$ ) using LSD test.

<sup>d</sup>> 5.50 indicates cells too numerous to count.

**Figure 2.1.** Total cells (■), CTC stained cells (▲), inactive cells (●) observed by direct microscopic observation and total CFU (◇) obtained using plated on CCA-KM of GFP-*Campylobacter* on the chicken skin (A), at 0-10µm under the surface (B), and at 20-30µm under the surface (C) after 0, 8, 16, and 24 hr of incubation at 25°C and after 0, 24, 28, 72, and 96 hr at 4°C. CTC stained *Campylobacter* cells were determined after subtracting the average number of total aerobic bacteria on the uninoculated control observed by DMC. This experiment was replicated three times.



## CHAPTER 3

### DIRECT MICROSCOPIC OBSERVATION IN EFFICACY OF SELECTED SANITIZERS FOR INACTIVATION OF *CAMPYLOBACTER JEJUNI* ON CHICKEN SKIN

---

<sup>1</sup>Chantarapanont, W., M. Berrang, and J. F. Frank. 2002. To be submitted to Journal of Food Protection



## ABSTRACT

The objective of this research was to determine the effectiveness of chlorine, acidified sodium chlorite and peracetic acid on *C. jejuni* viability and the relationship of viability to chicken skin microstructure. Confocal scanning laser microscopy (CSLM) was used to visualize *Campylobacter jejuni* transformed with  $P_{c}gfp$  plasmid (GFP-*Campylobacter*) exposed to 5-cyano-2,3-ditoly tetrazolium chloride (CTC) to detect viability along with plate count method. Inoculated chicken skins with 50 ppm for 2 min and 200 ppm for 15 min of each sanitizer were observed with CSLM. There was no significant difference in reducing *C. jejuni* on chicken skin among sanitizers applied at the same concentration and contact time. The effectiveness of these sanitizers in killing *C. jejuni* on chicken skin at different locations could not be differentiated in statistical analysis. When the number of live cells per microscopic field were determined, chlorine was the most effective in killing *C. jejuni* located at the surface of chicken skin but acidified sodium chlorite and peracetic acid were more effective than chlorine in killing *C. jejuni* at deeper locations. The detection of live cells after treatment by direct microscopic count at deeper locations has a limitation because of the low number of cells. The variation of results among replications was high due to the complexity of chicken skin attachment and the nature of *C. jejuni*. However, elimination *C. jejuni* from chicken skin was not achieved as shown by direct microscopic count and plate count.

*Campylobacter jejuni*, a common foodborne pathogen of humans, is associated with commercial broiler flocks and is found on processed poultry products (Jacobs-Rietsma, 2000). A large percentage of fresh and frozen poultry products are contaminated with *Campylobacter* (The National Advisory Committee on Microbiological Criteria for Foods, 1994). The intestinal tract of poultry serves as the reservoir of *Campylobacter* so fecal contamination on feathers and skin, leakage of fecal content, intestinal breakage are all possible causes of carcass contamination during processing, especially during defeathering and evisceration. Decontamination procedures during processing would be beneficial due to the failure of eliminating *Campylobacter* from poultry prior to processing.

U.S. commercial poultry processing currently uses chlorinated water chilling in circulating water tanks containing low levels of residual chlorine (up to 50ppm) at 4°C for approximately 1hr (Kemp, 2000). However, chlorine may corrode equipment and plant machinery and also generates mutagenic agents after reacting with organic materials (Masri, 1986). Another sanitizer, acidified sodium chlorite, was recently approved by FDA for use as a disinfectant in pre-chill and post-chill on whole carcasses and cut-up poultry parts (Kemp, 2002). A number of oxychlorous antimicrobial intermediates, which generated by acidified sodium chlorite, act as broad-spectrum germicides by breaking oxidative bonds on cell membrane surfaces (Kemp, 2000). Acidified sodium chlorite can also be corrosive to plant equipment and a health hazard to workers. Peracetic acid is widely used in food processing including poultry plants due to the absence of toxic residuals (Dychdala, 1988). With the highly reactive hydroxyl

radical, peracetic acid inhibits and kills gram positive and negative bacteria, fungi, and yeasts in 5 min or less at the concentration or less than 100ppm (Block, 2001).

Chicken skin protects and supports the survival of *C. jejuni* which is sensitive to various stresses including atmospheric oxygen (Lee, 1998). Without chicken skin, most common disinfecting agents at standard concentrations kill the microorganism (Wang, 1983). Our objective is to determine the effectiveness of chlorine, acidified sodium chlorite and peracetic acid on *C. jejuni* viability and the relationship of viability to chicken skin microstructure.

## MATERIALS AND METHODS

### **Bacterial strain and plasmid:** GFP *Campylobacter jejuni* RM 1221

(pWM1007) was provided by W. G. Miller USDA Agricultural Research Service, Albany, CA. The plasmid encoding GFP (pWM1007; Km<sup>r</sup>; pMW10 $\Delta$ lacZ $\Omega$ [(T1)<sub>4</sub>-P<sub>c</sub>-*gfp*-T1] had been mobilized into *C. jejuni* strain RM 1221 from a chicken carcass isolate (Miller et al., 2000). Transformants were selected by plating on Cefex-campy agar (CCA) (Stern, 1992) amended with 200  $\mu$ g of kanamycin per ml (CCA-KM) and grown at 42°C for 48 hr in sealed bag with a mixture of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub> (BOC Gases, Chattanooga, TN). Colonies on CCA-KM were restreaked on fresh plates, and expression of GFP-*Campylobacter* was confirmed by observation of bacterial smears with a Confocal Scanning Laser Microscope (CSLM) with the same setting as mention later. Kanamycin- resistant clones, which exhibited green fluorescent by microscopic

observation, were maintained at -70°C in 0.1% phosphate buffered saline (PBS) with sterile 20% glycerol.

**Culture Preparation:** Stock cultures of GFP-*Campylobacter* were resuscitated by streaking on CCA-KM and incubated at 42°C for 48 hr in sealed bag containing of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>. Cultures were transferred at least twice before using. For the attachment assay, a loopful of isolated colonies of GFP-*Campylobacter* on CCA-KM agar was suspended in sterile deionized water (SDW). The optical density (O.D.) of the cell suspension was adjusted to 0.5 – 0.6 using a spectrophotometer (Spectronic 21D; Milton Roy Co., Rochester, NY) at 540 nm to yield 10<sup>8</sup> - 10<sup>9</sup> cells/ml.

**Chicken skin:** Freshly processed broiler carcasses were randomly collected immediately upon exiting the chill tank in a commercial processing plant. Carcasses were individually bagged and stored in ice no more than 30 minutes before removal of breast skins. Breast skin (6 x 6 cm) was removed from the same location on breasts of broiler carcasses for each assay using sterile scalpel and forceps. Chicken skin was aseptically stored at -20°C, no more than one week before using.

**Attachment assay:** Attachment was determined as described by Kim et al (1996). Frozen chicken skins were thawed by rinsing 3 times for 3 min each with 15 ml of 4°C SDW on an orbital shaker model No. 3520 (Lab Line Instrument Inc, IL) at 100 rpm. Skins were then blotted dry under a laminar flow hood (SteriGard Hood, The Baker Company, Inc., Sanford, ME). The outer skin surface was placed toward the inside of a

sterile 2.5 cm-diameter bottomless centrifuge tube (Nalgene, CO.) and was kept in place with sterile rubber bands. Excess skin was trimmed and remaining skin was covered with aluminum foil to prevent contamination and drying. The tube with its bottom covered by skin was placed upright and a 3 ml suspension of  $10^8$ - $10^9$  cfu/ml of GFP-*Campylobacter* was added thereby exposing the outer surface of skin (at the bottom of the tube) to the bacteria. Attachment was allowed to take place at room temperature (21°C) while shaking on an orbital shaker at 70 rpm for 1 hr. The cell suspension was then decanted and unattached cells were removed by rinsing 3 times for 1 min each with 2 ml of SDW on an orbital shaker at 100 rpm. The negative control used the same procedure with 3 ml SDW instead of the suspension of GFP-*Campylobacter*.

**Sanitizer preparation and application:** Sanitizers used in this study included sodium hypochlorite (purified grade 4 to 6% NaOCl, Fisher Scientific, Pittsburgh, PA), peracetic acid (27.5% hydrogen peroxide, 5.8% peroxyacetic acid and 66.7% inert ingredients, Oxonia Active, Ecolab), and acidified sodium chlorite (Zep Seacide Tea Bag, Zep Company). The sanitizers were freshly prepared at concentrations of 40- and 100-ppm active agent before each use which calculated from ppm of active ingredient (in 1000ml of working solution) = (ml of product) x (%active ingredients) x 10. Sodium hypochlorite was diluted with 50mM potassium phosphate in deionized water adjusted to pH 7.2, peracetic acid was diluted with sterile deionized water, and acidified sodium chlorite was prepared as instruction of the company to give a concentration of 40 and 100 ppm. Free chlorine concentration was determined by spectrophotometric assay (Takeuchi et al., 2000). Total active ingredient concentrations (ppm by weight

percentage of total active ingredients) of peracetic acid were determined by using a Total Available Oxygen test kit (Ecolab, St. Paul, MN).

Pieces of GFP-*Campylobacter* inoculated chicken skins was individually immersed in 25 ml of each sanitizer for 2 min and 15 min and neutralized by immersion in an aqueous solution of 0.05% sodium thiosulfate-phosphate (Marshall, 1992). The same procedure was done with untreated inoculated chicken skin (positive control) and uninoculated chicken skin (negative control) using sterile distilled water.

**Microbiological Analysis:** After sanitizer treatment and neutralization, each treated and control chicken skin (two pieces per replication) was rinsed to remove unattached cells by manually shaking for 5s in a petri dish with 15 ml sterile water. Then skin samples were placed in sterile stomacher bag containing 5 ml of an enrichment broth medium, BR Tween, which contain 28 g/L brucella broth (Acumedia Manufactures, inc., Baltimore, MD), 1g/L Tween 80<sup>TM</sup> (Sigma), 33 mg/L cefoperazone, FBP (as described previously) and *Campylobacter* selective supplement (Bolton's) (Dalynn Biologicals, Calgary, Alberta, Canada) used as recommended by the manufacturer and massaged in a stomacher blender (Stomach<sup>®</sup> 80 Biomaster, Seward, England) at high speed for 2 min. Aliquots of the skin wash were serially diluted, spread plated in duplicate on CCA-KM, and incubated at 42°C 48 hr to enumerate GFP-*Campylobacter*. Five ml of phosphate buffer saline (PBS) was placed in sterile stomach bag with the negative control and the natural microflora on skin was enumerated by using plate count agar (PCA) incubated at 35°C for 24hr. Two pieces of inoculated chicken skin of each treatment and two positive controls were analyzed for each replication. Three replications were done. Data from

chicken skin was not used if the uninoculated control had more than 500 cfu of total aerobic bacteria in 1 cm<sup>2</sup>. Presumptive colonies of GFP-*Campylobacter* were confirmed by observing their motility under a phase contrast microscope (Lobophot, Nikon, Japan) and by a latex agglutination test (INDX-Campy<sup>TM</sup>, Integrated Diagnostics, Inc., Baltimore, MD).

**CTC staining:** CTC, 5-cyano-2,3-ditolyl tetrazolium chloride, (Polysciences, Warrington, PA) was diluted with R<sub>2</sub>A broth (Reasoner and Geldreich, 1985) to obtain a final concentration of 5 mM. One hundred and fifty microlitres of 5 mM CTC was applied to the skin sample and incubated in a dark chamber for 30 min at room temperature. CTC-stained skin was then rinsed with 15 ml SDW 3 times (1 min each) on an orbital shaker at 100 rpm. A 1x1-cm specimen was cut from the middle of skin sample for microscopic analysis.

**Visualization of GFP-*Campylobacter* on chicken skin:** Cover well imaging chamber gaskets (Molecular Probes, Eugene, OR) of 2 mm thickness were used to prevent compression of the chicken skin during microscopic observation. A drop of 50% glycerol in PBS was placed into the chamber as a mounting medium. The outer surface of specimen was placed upside down on mounting medium and then securely sealed within the chamber by placing a microscope slide against the gasket surface and pressing gently around the edges of the slide. At least ten microscopic fields were randomly scanned for each specimen. The metabolic active and inactive GFP-*Campylobacter* was determined at different depths from the surface.

**Confocal Scanning Laser Microscope (CSLM):** A TCS NT SP2 Leica

Confocal microscope (Leica Microsystems Heidelberg GmbH, Germany) equipped with a 40x (numerical aperture = 1.25, Leica, Germany) and 100x oil immersion objective (numerical aperture = 1.3, Leica, Germany) was used for microscopic observation. An Ar laser (excitation wavelength [ $\lambda$ ] = 488 nm) was used to excite GFP and CTC-formazan. Emitted light was collected through a triple dichroic mirror (TD) 488/568/633. The reflected light (wavelength of 483-495 nm) was assigned as a grey color for chicken skin-reflected light, emitted light (495-540 nm) as green color for GFP image, and 600-670 nm emission as red color for CTC-formazan image. TCS NT software (version 1.6.551; Leica Microsystems) and Adobe Photoshop version 6.0 (Adobe Systems Incorporated, US) were used to process the images. Because of nonselective staining of CTC and autofluorescence of chicken skin, uninoculated chicken skin, stained with and without CTC were used to calibrate the sensitivity of photomultipliers for detecting GFP-*Campylobacter* and CTC-formazan to reduce noise before scanning. ImageJ 1.27z (National Institutes of Health, USA) was used for image analysis.

**Statistical analysis:** A 3 x 2 x 2 factorial design in a randomized completed block design with three replications. The main effects were sanitizers (peracetic acid, acidified sodium chlorite, and chlorine), treatment concentration (40 and 100 ppm), and treatment time (2 and 15 min). For CSLM observation, inoculated chicken skin treated with 40 ppm for 2 min and 100 ppm for 15 min of each sanitizer were observed. Two pieces of inoculated skin were used for CTC-stained for each treatment, making a total of



6 pieces after three replications. Data was analyzed with SAS software (SAS Institute, Cary, NC) using PROC ANOVA. Significant differences between means were determined using Least Significant Difference (LSD) test. Significance was determined the 95% confidence level ( $P = 0.05$ ).

## RESULTS AND DISCUSSION

The ability of sanitizers to reduce levels of *C. jejuni* on chicken skin is shown in Figure 3.1. Sanitizers reduced *C. jejuni* on the chicken skin by similar amounts, when applied on chicken skin at the same concentration and contact time. Increasing concentration of sanitizer or contact time lead to an increased reduction of *C. jejuni*. All sanitizers reduced *C. jejuni* on chicken skin the most when applied at 100ppm for 15min and the least when applied at 40ppm for 2 min (Fig. 3.1). There was no significant difference whether application was at 40ppm for 15min or 100ppm for 2 min. When submerging inoculated chicken skin in 40ppm of acidified sodium chlorite and peracetic acid for 15min, the effectiveness in reduction *C. jejuni* on chicken skin was greater than that observed by increasing concentration to 100ppm but with 2 min. When leaving chicken skin in 40ppm of chlorine from 2 to 15min, the reduction of *C. jejuni* did not increase as much as when acidified sodium chlorite or peracetic acid were allowed longer exposure. The effectiveness of acidified sodium chlorite and peracetic acid increased with longer exposure at both low and high concentration. At the same contact time (2 or 15 min), increasing concentration of sanitizer increases reduction of *C. jejuni* on chicken skin at approximately same proportion (Fig. 3.1).

**Direct observation on effect of sanitizers.** Cells that are metabolically active are able to reduce CTC to CTF, which fluoresces in red color. Green fluorescence from GFP-*C. jejuni* cells present on chicken skin with CTC staining indicates inactive cells, which are unable to reduce CTC. The emission wavelength of green fluorescence from GFP-*C. jejuni* (495-540 nm with 488 nm Ar laser excitation) is within the wavelength that is able to excite CTF to fluorescence (less than 400 nm to more than 550 nm) in the same cells or green fluorescence of GFP-*Campylobacter* is degraded due to cell death. This may have occurred when cells were exposed to CTC. Most of green fluorescent *C. jejuni* cells are not detected at the same location as the red cells. So the total number of cells will be the sum of live and inactive cells. The average number of GFP-*C. jejuni* on chicken skin after inactivation with different sanitizers by using direct microscopic count and enumerating on CCA-KM is shown in Table 3.1. Live cell counts obtained from direct microscopic counts were similar to counts obtained by CCA-KM (Table 3.1). There was no significant difference between these two methods in detecting viable *C. jejuni* on chicken skin. The direct microscopic count method was more sensitive than plate counts when detecting viable cells after treatment with high concentration of sanitizer. It is possible that injured cells cannot form colonies on CCA-KM. Population of *C. jejuni* on chicken skin enumerated on CCA-KM and from direct microscopic count significantly decreased after treatment with sanitizers at 100ppm for 15 min. At 100 ppm and 15 min contact time, acidified sodium chlorite and chlorine was the most effective sanitizer, which can reduce *C. jejuni* by 1.16 log<sub>10</sub> and 0.72 log<sub>10</sub>, as determined by plate count and direct microscopic count, respectively. However, there was no significant difference between acidified sodium chlorite and chlorine in reduction of live *C. jejuni* on

chicken skin at the same application, obtained by both plate count and microscopic count method. Number of total GFP-*Campylobacter* cells decreased after sanitizing, although there was no significant difference in total GFP-*C. jejuni* cells on chicken skin after applying sanitizers when compare with untreated control.

Total number of live cells on chicken skin located on 0-10  $\mu\text{m}$ , 10-20  $\mu\text{m}$ , and 20-30  $\mu\text{m}$  are presented in Tables 3.2.1, 3.2.2, and 3.2.3, respectively. Detection of live *C. jejuni* after treatment at below surface was difficult due to low number of cells present at these locations, so the results were reported as the combination of three replications. The greatest reduction of all sanitizers occurred at the surface (0-10  $\mu\text{m}$ ) where live cells were mostly present (Table 3.2.1). Live cells were detected in all depths after sanitizing although there were fewer at below the surface. At one location, *C. jejuni* at the surface and at 20-30  $\mu\text{m}$  was completely inactivated by acidified sodium chlorite but not at 0-10  $\mu\text{m}$  (Fig 3.2). When number of live cells per microscopic field were determined, chlorine 100 ppm applied for 15 min was the most effective in killing *C. jejuni* at the 0-10  $\mu\text{m}$  depth (Table 3.2.1). Acidified sodium chlorite and peracetic acid were more effective than chlorine in killing *C. jejuni* at 10-20 and 20-30  $\mu\text{m}$  depth but required a longer contact time (Table 3.2.2 and Table 3.2.3).

Chemical disinfectants vary in their ability to kill microorganisms. Organic materials and high pH reduce chlorine's ability as a disinfectant (Block, 2001). Acidified sodium chlorite and peracetic acid are less affected by organic matter than is active chlorine, but they often act more slowly (Fatemi and Frank, 1999). Chicken skin is high in organic matter which may affect the effectiveness of chlorine when applied in low concentration. To achieve the most effective antimicrobial effect of chlorine, high

volume of sanitizer solution and sufficient concentration is needed. Trachoo and Frank (2002) reported that peracetic acid was not as effective as chlorine against *C. jejuni* in biofilm. Due to the limited ability of chlorine to penetrate extra cellular polymeric substances, *C. jejuni* might be located close to the surface or that the biofilms were not sufficiently thick to provide protection. Our data showed that there was no significant difference among all sanitizers in killing *C. jejuni* on chicken skin. Live cells after treatment with chlorine was less than after treatment with other sanitizers obtained by direct microscopic count. This might be because most of live cells on chicken skin located at the surface or due to a limitation of direct microscopic count in detection *C. jejuni* below the surface. It is also possible that *C. jejuni* can survive deeper than 30  $\mu\text{m}$  in chicken skin, which was excluded from detection by direct microscopic count. Acidified sodium chlorite and peracetic acid showed more reduction of *C. jejuni* on chicken skin obtained by plate count method. The concentration of sanitizer, contact time, and application method are factors necessary to achieve the most effective.

Chlorine, acidified sodium chlorite and peracetic acid all generate oxidizing agents. Acid in acidified sodium chlorite acts as a proton donor to permit acidification of sodium chlorite and influences the pH affecting the rate of dissociation of the chlorite to form the antimicrobial chlorous acid species (Kemp et al, 2000). The benefit of acid in acidified sodium chlorite in killing *C. jejuni* at the surface of chicken skin was not clearly seen here due to low concentration used. Kemp et al (2000) showed that a 5-s exposure of acidified sodium chlorite at 1,200 ppm was more effective than exposure of 20 ppm chlorinated water for 1 hr or more.

In conclusion, the effect of chlorine, acidified sodium chlorite and peracetic acid on *C. jejuni* viability on chicken skin at different locations could not be differentiated by statistical analysis. These sanitizers showed no difference in ability to kill *C. jejuni* on chicken skin. High variation of results among replications was observed due to the complexity of chicken skin and the nature of *C. jejuni* attachment. The detection of live cells at deeper locations by direct microscopic count was limited by low cell numbers. However, complete elimination of *C. jejuni* from chicken skin was not achieved as shown by direct microscopic count and plate count.

#### **ACKNOWLEDGEMENTS**

This research was supported by State and Hatch funds allocated to the Georgia Agricultural Experiments stations and the Royal Thai Government.

## REFERENCES

- Block, S. S. 2001. Peroxygen Compounds. *In* Disinfection, Sterilization, and Preservation. S.S. Block. (eds). Lippincott Williams & Wilkins. Philadelphia, PA. p. 185-204
- Dychdala, G. R. 1988. New hydrogen peroxide-peroxyacetic acid disinfectant. *In* Proceedings of the 4th conference on progress in chemical disinfection. Binghamton, NY.
- Fatemi, P. and J. F. Frank. 1999. Inactivation of *Listeria monocytogenes*/ *Pseudomonas* biofilms by peracid sanitizers. *J. Food Prot.* 62:761-765.
- Jacobs-Reitsma, W. 2000. *Campylobacter* in the food supply. *In* *Campylobacter*. I. Nachamkin and M.J. Blaser (eds). ASM Press. Washington, DC. p. 467-481
- Kemp, G., M. L. Aldrich, and A. L. Waldroup. 2000. Acidified sodium chlorite antimicrobial treatment of broiler carcasses. *J. Food Prot.* 63:1087-1092.
- Kemp, G. and K. R. Schneider. 2002. Reduction of *Campylobacter* contamination on broiler carcasses using acidified sodium chlorite. *Dairy, Food and Environmental Sanitation.* 22(8):599-606.
- Kim, K. Y., H. S. Lillard, J. F. Frank, and S. E. Craven. 1996. Attachment of *Salmonella typhimurium* to poultry skin as related to cell viability. *J. Food Sci.* 61(2): 439-441.
- Lee, A., S. C. Smith, et al. 1998. Survival and growth of *Campylobacter jejuni* after artificial inoculation onto chicken skin as a function of temperature and packaging conditions. *J. Food Prot.* 61(12): 1609-1614.
- Marshall, R. T. 1992. Standard methods for the examination of dairy products. Vol. 16 American Public Health Association. Washington, D. C.
- Masri, M., 1986. Chlorinating poultry chiller water: the generation of mutagens and water re-use. *Food Chem. Toxicol.* 24:923-930.
- Miller, W. G., A. H. Bates, S. T. Horn, M. T. Brandl, M. R. Wachtel, and R. E. Mandrell. 2000. Detection on surfaces and in caco-2 cells of *Campylobacter jejuni* cells transformed with new *gfp*, *yfp*, and *cfp* marker plasmids. *Appl. Environ. Microbiol.* 66(12): 5426-5436.

- Reasoner, D. J. and E. E. Geldrich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* 49: 1-7.
- Stern, N. J., B. Wojton, and K. Kawiatek. 1992. A differential selective medium and dry ice generated atmosphere for recovery of *Campylobacter jejuni*. *J. Food Prot.* 55: 514-517.
- Takeuchi, K. and J. F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. *J. Food Prot.* 63(4): 434-440.
- The National Advisory Committee on Microbiological Criteria for Foods. 1994. *Campylobacter jejuni/coli*. *J. Food Prot.* 57:1101-1121.
- Trachoo, N. and J. F. Frank. 2002. Effectiveness of chemical sanitizers against *Campylobacter jejuni*-containing biofilms. *J. Food Prot.* 65(7): 1117-1121.
- Wang, W. L., B. W. Powers, N. W. Luechterfeld, and M. J. Blaser. 1983. Effects of disinfectant on *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 45(4):1202-1205.

**Table 3.1.** Average population of *C. jejuni* on chicken skin after treatment with sanitizers at different concentrations and contact times. Enumeration was on CCA-KM after incubation at 42°C for 48 hr and by direct microscopic count using CTC staining.



Sanitizer	Concentration (ppm)	Time (min)	GFP- <i>C. jejuni</i> Population on chicken skin (log cfu/cm <sup>2</sup> )			
			CCA-KM <sup>a</sup>	Direct Microscopic Count		
				Live Cells <sup>b</sup>	Inactive cells <sup>c</sup>	Total cells <sup>d</sup>
No treatment			A5.44±0.6a <sup>e</sup>	A5.51±0.6a	A6.06±0.5a	A6.19±0.5a
Chlorine	40	2	AB5.04±0.1b	AB4.97±0.1b	A5.76±0.1a	A5.83±0.8a
	100	15	BCD4.48±0.4b	B4.79±0.5b	A5.78±0.2ab	A5.83±0.2a
Acidified Sodium Chlorite	40	2	ABC5.03±0.1b	AB4.97±0.3b	A5.62±0.2a	A5.72±0.1a
	100	15	D4.28±0.4b	B4.84±0.4b	A5.62±0.4a	A5.69±0.3a
Peracetic acid	40	2	ABC5.02±0.2bc	AB4.87±0.5c	A5.63±0.4ab	A5.73±0.3a
	100	15	CD4.39±0.4b	B4.83±0.1b	A5.84±0.3a	A5.89±0.3a

<sup>a</sup>*C. jejuni* was enumerated on Campy Cefex Agar with 200 mg/l Kanamycin at 42°C for 48 hr.

<sup>b</sup>Live cells were red CTC stained cells.

<sup>c</sup>Inactive cells were cells that fail to reduce CTC (green fluorescence).

<sup>d</sup>Total cells were the sum of live cells and inactive cells.

<sup>e</sup>Values were the means of log cfu/cm<sup>2</sup> of GFP-*C. jejuni* population on chicken skin and standard deviation from three replications. Different capital letters in the same column and different small letters in the same row indicate significant differences ( $P<0.05$ ) using LSD test.

**Table 3.2.1.** Total number of live *C. jejuni* present at 0-10  $\mu\text{m}$  depth on chicken skin after treatment with sanitizers.

		Sanitizers					
		Chlorine		Acidified sodium chlorite		Peracetic acid	
		Concentration (ppm)					
		40	100	40	100	40	100
		Time (min)					
		2	15	2	15	2	15
Number of live cells <sup>a</sup> (cells)	549	117	98	151	112	139	125
Number of microscopic fields <sup>b</sup> (fields)	39	35	37	38	35	38	39
Number of live cells/ field	14.08	3.34	2.65	3.97	3.2	3.66	3.21

<sup>a</sup>Number of live cells was the sum of live cells in three replications.

<sup>b</sup>Number of microscopic fields was the sum of microscopic fields in three replications. The area of a microscopic field was  $2.25 \times 10^4 \mu\text{m}^2$ .

**Table 3.2.2.** Total number of live *C. jejuni* present at 10-20  $\mu\text{m}$  depth on chicken skin after treatment with sanitizers.

		Sanitizers					
		Chlorine		Acidified sodium chlorite		Peracetic acid	
		Concentration (ppm)					
		40	100	40	100	40	100
		Time (min)					
		2	15	2	15	2	15
Number of live cells <sup>a</sup> (cells)	157	16	13	27	16	41	9
Number of microscopic fields <sup>b</sup> (fields)	34	24	31	28	27	28	32
Number of live cells/ field	4.62	0.67	0.42	0.96	0.59	1.46	0.28

<sup>a</sup>Number of live cells was the sum of live cells in three replications.

<sup>b</sup>Number of microscopic fields was the sum of microscopic fields in three replications. The area of a microscopic field was  $2.25 \times 10^4 \mu\text{m}^2$ .

**Table 3.2.3.** Total number of live *C. jejuni* present at 20-30  $\mu\text{m}$  depth on chicken skin after treatment with sanitizers.

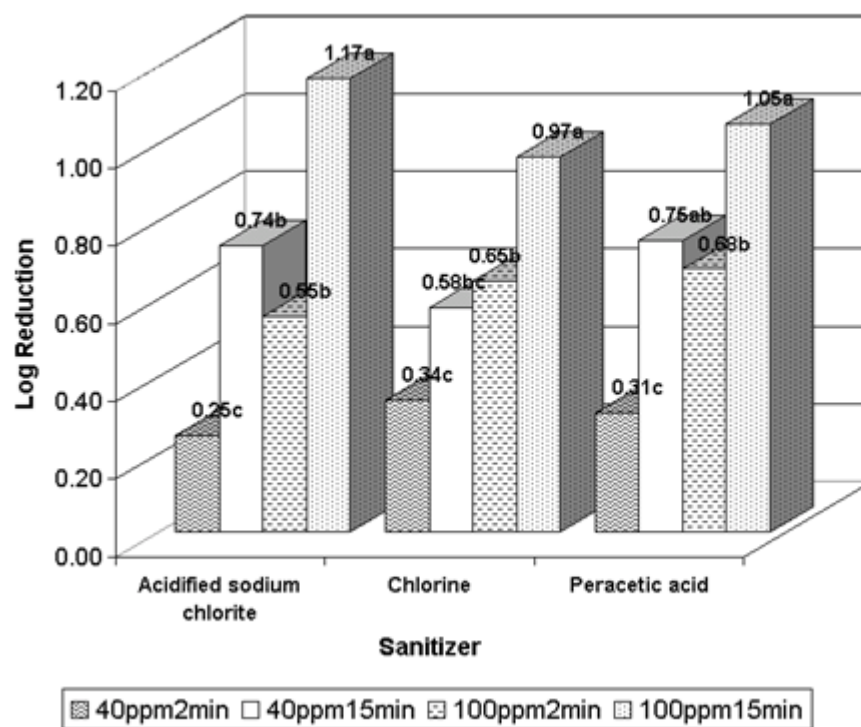
		Sanitizers					
		Chlorine		Acidified sodium chlorite		Peracetic acid	
		Concentration (ppm)					
		40	100	40	100	40	100
		Time (min)					
		2	15	2	15	2	15
Number of live cells <sup>a</sup> (cells)	85	6	7	11	4	7	1
Number of microscopic fields <sup>b</sup> (fields)	23	12	17	17	12	19	15
Number of live cells/ field	3.69	0.50	0.41	0.65	0.33	0.67	0.37

<sup>a</sup>Number of live cells was the sum of live cells in three replications.

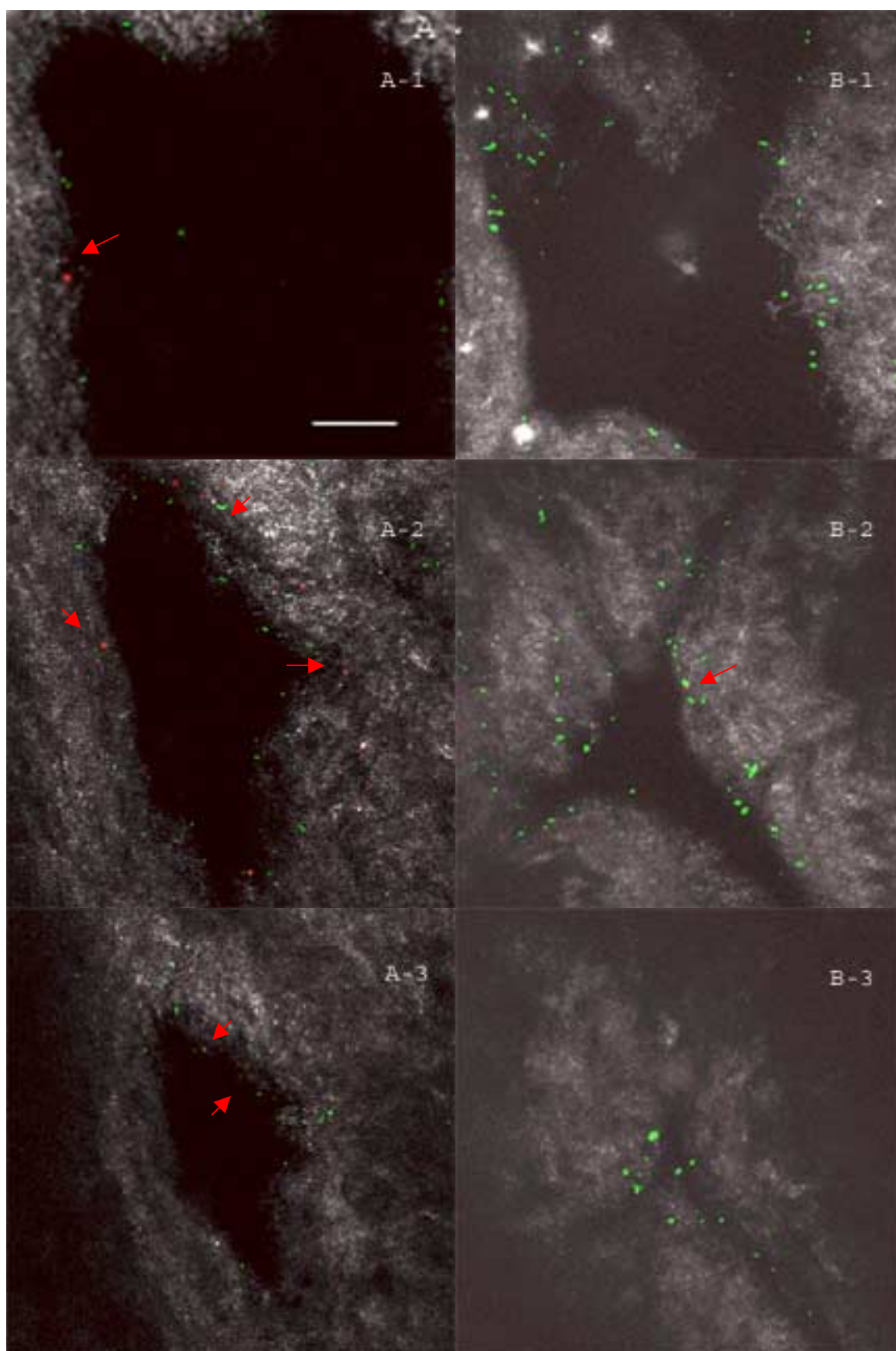
<sup>b</sup>Number of microscopic fields was the sum of microscopic fields in three replications. The area of a microscopic field was  $2.25 \times 10^4 \mu\text{m}^2$ .

**Figure 3.1.** Average log reduction of GFP-*C. jejuni* population on chicken skin determined on CCA-Km after applying different sanitizers at different conditions. Values are the means and different letters in the same sanitizer indicate significant differences at ( $P < 0.05$ ) using LSD test. The  $\log_{10}$  reduction is the difference between the average  $\log_{10}$  population of GFP-*C. jejuni* on chicken skin after treated with sterile water (positive control) and each treatment at the same condition.





**Figure 3.2.** GFP-*Campylobacter* with CTC staining showing the effect of 100ppm acidified sodium chlorite treatment on the viability of *C. jejuni* cells entrapped in feather follicle at different depths. Live GFP-*Campylobacter* stained with CTC showing red cells which indicated by red arrows. Micrograph A and B represent 0-10, 10-20, and 20-30  $\mu\text{m}$  depth, respectively. Micrograph 1 represented untreated inoculated sample with CTC stained and Micrograph 2 represented 100ppm acidified sodium chlorite treated sample for 15min and stained with CTC. Each micrograph combined data from 10 Optical sections, which were collected using 1- $\mu\text{m}$  increments. Scale bar for each micrograph = 20 $\mu\text{m}$ .



## CONCLUSION

Visualization of *Campylobacter jejuni* transformed with P<sub>c</sub>*gfp* plasmid (GFP-*Campylobacter*) exposed to 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) by confocal laser scanning laser microscopy (CSLM) can be used as a tool to detect total and active *Campylobacter* at various sites on chicken skin. Viability counts obtained using CTC staining must be interpreted with caution since all viable cells may not reduce the stain under the experimental conditions, and because nonspecific reduction may make it difficult to discriminate the target bacterial population. When viability detection of *C. jejuni* was observed on chicken skin stored at 25°C and 4°C, the natural microflora on chicken skin also reduced CTC, observation of viable cells could be obtained only up to 8 hr for samples were stored at 25°C and up to 72 hr at 4°C, after which time the natural microflora overgrew the campylobacter. To use this method to detect of live cells after sanitizing at deeper location, the presentation in a low number of cells makes it difficult to detect viable cells at deep location.

The observation showed that GFP-*Campylobacter* remaining on the chicken skin surface after rinsing were mostly located in crevices, entrapped inside feather follicles with water and also entrapped in the surface water layer. Most of viable cells were entrapped with water in the skin crevices and feather follicles where provide suitable microenvironment for GFP-*Campylobacter* to survive. During storage at 4 and 25°C, live and dead campylobacters are initially retained with water on the skin and penetrate

into the skin with time. Chicken skin provides a suitable environment for campylobacter to survive and grow at 25°C and 4°C, respectively. Complete elimination of *C. jejuni* from chicken skin by sanitizing was not achieved as shown by direct microscopic count and plate count.