การปนเปื้อนของเชื้อแคมไพโลแบคเตอร์ที่แยกได้จากไก่เนื้อในระหว่างกระบวนการผลิตในโรงฆ่าสัตว์ปีก

นางสาวสุธิดา ม่วงน้อยเจริญ

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CONTAMINATION OF CAMPYLOBACTER ISOLATES FROM BROILERS DURING SLAUGHTERING PROCESS

Miss Suthida Muangnoicharoen

A Thesis Submitted in Partial Fulfillment of the Requirements
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By	Miss Suthida Muangnoicharoen
Field of Study	Veterinary Public Health
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Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science
(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Alongkorn Amonsin, D.V.M., Ph.D.)

.....Thesis Advisor
(Taradon Luangtongkum, D.V.M., Ph.D.)

.....Examiner
(Associate Professor Rungtip Chuanchuen, D.V.M., M.Sc., Ph.D.)

.....Examiner
(Associate Professor Suphachai Nuanualsuwan, D.V.M., M.P.V.M., Ph.D.)

.....External Examiner
(Associate Professor Pravate Tuitemwong, Ph.D.)

สุธิดา ม่วงน้อยเจริญ : การปนเปื้อนของเชื้อแคมไพโลแบคเตอร์ที่แยกได้จากไก่เนื้อ ในระหว่างกระบวนการผลิตในโรงฆ่าสัตว์ปีก (CONTAMINATION OF CAMPYLOBACTER ISOLATES FROM BROILERS DURING SLAUGHTERING PROCESS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. น.สพ. ดร.ธราดล เหลืองทองคำ, 58 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจสอบอัตราการปนเปื้อนของเชื้อแคมไพโลแบคเตอร์ ปริมาณของเชื้อ และลักษณะทางพันธุกรรมของเชื้อที่แยกได้จากซากไก่ในระหว่างกระบวนการผลิตในโรงฆ่าสัตว์ปีก 3 แห่ง ระหว่างเดือนมิถุนายน 2555 ถึงเดือนเมษายน 2556 จำนวนทั้งสิ้น 320 ตัวอย่าง ซึ่งประกอบด้วย สวอปจากทวารหนักของไก่เนื้อ 40 ตัวอย่าง น้ำล้างซากไก่ในระหว่างขั้นตอนต่างๆ ของกระบวนการผลิต จำนวน 5 ขั้นตอน ได้แก่ หลังลวก หลังถอนขน หลังล้วงเอาเครื่องในออก หลังล้างภายในและภายนอกซาก และหลังลดอุณหภูมิซาก รวมทั้งหมด 200 ตัวอย่าง และไส้ตัน 80 ตัวอย่าง อัตราการปนเปื้อนตรวจสอบด้วยวิธี direct plating และวิธี selective enrichment ร่วมกัน ในขณะที่ปริมาณของเชื้อที่ปนเปื้อนตรวจสอบด้วยวิธี direct plating การจำแนกลักษณะทางพันธุกรรมของเชื้อที่แยกได้ใช้วิธี *flaA*-short variable region (*flaA*-SVR) sequencing จากการเก็บตัวอย่างไก่เนื้อที่มีประวัติการติดเชื้อแคมไพโลแบคเตอร์ในฟาร์มจำนวนทั้งหมด 8 ฝูง ผลการศึกษาพบว่าอัตราการปนเปื้อนของเชื้อแคมไพโลแบคเตอร์ในซากไก่ ไส้ตัน และสวอปทวารหนัก อยู่ที่ร้อยละ 74.5 73.6 และ 62.5 ตามลำดับ อัตราการปนเปื้อนของเชื้อในซากไก่หลังลวกเท่ากับร้อยละ 50 อัตราการปนเปื้อนของเชื้อเพิ่มขึ้นสูงสุดในขั้นตอนหลังถอนขน (ร้อยละ 85) และหลังล้วงเอาเครื่องในออก (ร้อยละ 82.5) การปนเปื้อนยังคงสูงหลังล้างภายในและภายนอกซาก (ร้อยละ 80) และหลังแช่ลดอุณหภูมิซาก (ร้อยละ 75) ปริมาณเชื้อโดยเฉลี่ยในซากไก่หลังลวกเท่ากับ 1.88 CFU/ml โดยปริมาณเชื้อโดยเฉลี่ยสูงขึ้นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ในขั้นตอนถอนขนและล้วงเอาเครื่องในออก ซึ่งเท่ากับ 2.76 และ 3.26 log CFU/ml ตามลำดับ ปริมาณเชื้อยังคงสูงหลังล้างภายในและภายนอกซาก ซึ่งพบปริมาณเชื้อโดยเฉลี่ยเท่ากับ 3.42 log CFU/ml หลังจากแช่ลดอุณหภูมิซาก พบว่าจำนวนเชื้อโดยเฉลี่ยลดลงอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เท่ากับ 2.04 log CFU/ml โดยส่วนใหญ่การปนเปื้อนของเชื้อที่พบในแต่ละฝูงลดลงทั้งในโรงฆ่าสัตว์ที่ใช้คลอรีนและไม่ใช้คลอรีนในการลดอุณหภูมิซาก ลักษณะทางพันธุกรรมหลักที่พบในการศึกษาครั้งนี้ได้แก่ *flaA*-SVR allele 208 287 769 และ783 โดยลักษณะทางพันธุกรรมของเชื้อที่แยกได้จากน้ำล้างซากไก่สอดคล้องกับลักษณะทางพันธุกรรมของเชื้อที่แยกได้จากไส้ตันและสวอปทวารหนัก การศึกษาครั้งนี้สรุปได้ว่าการปนเปื้อนของเชื้อแคมไพโลแบคเตอร์สู่ซากไก่มักเกิดขึ้นในขั้นตอนการถอนขน รวมถึงขั้นตอนการล้วงเอาเครื่องในออก ดังนั้นการวางแนวทางเพื่อลดการปนเปื้อนของเชื้อแคมไพโลแบคเตอร์ในขั้นตอนการถอนขนจึงจำเป็นเพื่อควบคุมการปนเปื้อนของเชื้อสู่ซากไก่ในระหว่างกระบวนการผลิต นอกจากนั้นแล้วการควบคุมการปนเปื้อนของเชื้อสู่ซากไก่ในขั้นสุดท้ายยังทำได้ค่อนข้างยาก หากฝูงไก่นั้นมีการติดเชื้อแคมไพโลแบคเตอร์จำนวนมาก ดังนั้นการวางมาตรการเพื่อป้องกันการติดเชื้อและการแพร่กระจายของเชื้อแคมไพโลแบคเตอร์ในฟาร์มจึงสำคัญและควรรีบดำเนินการอย่างเร่งด่วน

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SUTHIDA MUANGNOICHAROEN: CONTAMINATION OF CAMPYLOBACTER ISOLATES FROM BROILERS DURING SLAUGHTERING PROCESS. ADVISOR: TARADON LUANGTONGKUM, D.V.M., Ph.D., 58 pp.

The objective of the present study was to determine contamination rates, concentration and genotypes of *Campylobacter* on broiler carcasses during slaughtering processes. A total of 320 samples including cloacal swabs (n=40), carcass rinses during slaughtering process (n=200) and ceca (n=80) from 8 *Campylobacter* positive broiler farms were collected from 3 slaughterhouses during June 2012 to April 2013. Carcass rinses were taken after scalding, defeathering, evisceration, inside-outside (I/O) washing and chilling steps. To determine *Campylobacter* contamination rates, direct plating method and selective enrichment method were performed, while the concentration of *Campylobacter* on chicken carcasses was detected by direct plating method. In addition, genotyping of *Campylobacter* isolates was carried out by *flaA*-short variable region (*flaA*-SVR) sequencing. The occurrence of *Campylobacter* in carcass rinse, cecum and cloacal swab was 74.5%, 73.6% and 62.5%, respectively. Our finding revealed high *Campylobacter* contamination rates after defeathering (85%) and evisceration (82.5%) which was higher than after scalding (50%). The contamination rate remained high after I/O washing (80%) and chilling (75%). The mean concentration of *Campylobacter* on carcasses after scalding was 1.88 log CFU/ml. *Campylobacter* concentration significantly increased ($p<0.05$) after defeathering (2.76 log CFU/ml) and evisceration (3.26 log CFU/ml). Mean concentration was highest after I/O washing (3.42 log CFU/ml) and significantly declined to 2.04 log CFU/ml after chilling ($p<0.05$). Reduction in the concentration of *Campylobacter* on post-chill carcasses was found in all slaughterhouses; with or without the use of chlorine during chilling step. *FlaA*-SVR types obtained during slaughtering process were different among broiler flocks. The most common allele types identified among *Campylobacter* isolates in this study were *flaA*-SVR allele 208, 287, 769 and 783. *FlaA*-SVR types recovered from carcass rinse during slaughter were mostly related to allele types present in cecum and cloacal swab. Since defeathering was considered as a crucial step, aside from evisceration, for *Campylobacter* contamination on chicken carcasses during slaughter, the implementation of measures to reduce *Campylobacter* contamination during defeathering is necessary for controlling *Campylobacter* contamination at the slaughter level. Additionally, if chickens enter slaughterhouse with high loads of *Campylobacter,* it will be almost impossible to get rid of this foodborne pathogen from fully processed carcasses. Therefore, intervention strategies to prevent the introduction of *Campylobacter* into broiler farms are required and should be urgently investigated.

Department:	Veterinary Public Health	Student's Signature	.....
Field of Study:	Veterinary Public Health	Advisor's Signature	.....
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CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION AND SYMBOLS

C.	*Campylobacter*
bp	base pair (s)
BPW	buffered peptone water
℃	degree (s) Celsius
CFU	colony-forming unit
DNA	deoxyribonucleic acid (s)
EU	the European Union
EFSA	European Food Safety Authority
et al.	et alibi and others
flaA-SVR	*flaA* Short Variable Region
g	gram (s)
h	hour (s)
ISO	International Organization for Standardization
mCCDA	modified Charcoal Cefoperazone Deoxycholate Agar
min	minute (s)
ml	milliliter (s)
PCR	polymerase chain reaction
SE	standard error

sec	second (s)
spp.	species
TAE	Tris-Acetate EDTA
µl	micro liter (s)
USDA	U.S. Department of Agriculture
v/v	volume per volume
w/v	weight per volume

CHAPTER I

INTRODUCTION

Campylobacter is one of the major causes of human gastrointestinal disease in many countries. Illness caused by *Campylobacter* can vary from mild watery diarrhea to serious complications including acute paralysis of the peripheral nervous system known as Guillain-Barré Syndrome. *Campylobacter* infection in humans has been frequently reported in many countries especially in developed countries (Stern, 2001). The European Food Safety Authority (EFSA) stated that campylobacteriosis was the most common foodborne disease in European Union with 220,209 confirmed human cases in 2011 (EFSA, 2013). Similarly, *Campylobacter* infection is the second most frequently reported foodborne illness in the United States. The US Centers for Disease Control and Prevention revealed 14.3 *Campylobacter* infection cases reported per 100,000 population in 2012 (CDC, 2013). Due to the high incidence of foodborne campylobacteriosis, the contamination of this pathogen in food system is a concern.

Diarrhea caused by *Campylobacter* is mainly associated with consumption of undercooked poultry meat. Previous studies have shown that poultry play an important role as a reservoir for *Campylobacter* transmission to humans (Baker et al., 2006; Wong et al., 2007; Sheppard et al., 2009a). *Campylobacter* can contaminate chicken meat during slaughter; therefore, several studies have been conducted to determine the occurrence and concentration of *Campylobacter* in chickens during slaughtering process (Stern and Robach, 2003; Allen et al., 2007; Berrang et al., 2007; Son et al., 2007; Figueroa et al., 2009; Hue et al., 2010). High level of *Campylobacter* contamination in retail chicken carcasses increases the possibility of illness caused by

this pathogen. Hence, the reduction of *Campylobacter* contamination in chicken would have significant impact on the incidence of *Campylobacter* infection in humans.

Since contamination level of *Campylobacter* on chicken carcasses during slaughtering has direct effects on the load of this bacterium on retail chicken meat, it is important to determine which steps in processing line influence the contamination of *Campylobacter*. Moreover, determination of genetic profiles of *Campylobacter* contaminating on chicken carcasses would provide better understanding about the contamination of *Campylobacter* during slaughtering process. In order to fully understand the changes in contamination level and strains of *Campylobacter* during slaughtering process, the study on the occurrence, concentration and genetic profiles of *Campylobacter* isolates from chicken carcass must be performed. Although contamination rates and genetic profiles of *Campylobacter* in Thai poultry slaughterhouses were reported in some studies (Saengthongpinit et al., 2010; Osiriphun et al., 2011; Chokboonmongkol et al., 2013), the changes of the contamination level and strains of *Campylobacter* along the whole processing line has not been fully investigated. Effective control strategies to reduce *Campylobacter* contamination would not be accomplished without the full knowledge of *Campylobacter* contamination along the slaughtering line. Therefore, the purpose of this study was to determine the contamination level and strains of *Campylobacter* on Thai broiler carcasses during slaughtering processes.

CHAPTER II

LITERATURE REVIEW

2.1 General characteristics of *Campylobacter* spp.

The genus *Campylobacter* belongs to the family *Campylobacteriaceae* and consists of 18 species and subspecies including *Campylobacter jejuni* and *Campylobacter coli* and so on. *Campylobacter* is a motile, spiral rod, Gram negative bacterium. The size of this bacterium is approximately 0.2-0.8 μm wide and 0.5-5 μm long. *Campylobacter* is microaerophilic and highly sensitive to drying and freezing conditions (Smibert, 1978). This organism is called thermophilic *Campylobacter* because it can grow at temperature between 37 to 42°C. The morphological characterization of *Campylobacter* colonies on agar plates is grey, moist and flat. *C. jejuni* may be found as thin spreading colonies especially on moist agar and sometimes was seen as metallic sheen, while the colonies of *C. coli* tend to appear as creamy, grey, moist and slightly raised (Corry, 2012). In terms of biochemical reaction, *Campylobacter* is positive to oxidase test and catalase test but only *C. jejuni* is hippurate hydrolysis positive (Hunt et al., 2001).

Campylobacter is mostly found in the intestinal tract of domestic and wild animals. This organism mainly inhabits in the intestinal tract of poultry including commercial broilers. However, it rarely causes diseases in chickens.

2.2 *Campylobacter* in humans and its transmission to humans

Campylobacter can cause an illness called campylobacteriosis in humans. Symptoms of *Campylobacter* infection include diarrhea, abdominal pain, fever, headache, nausea and vomiting. Symptoms can start from 1–10 days after infection, and usually last for 3–6 days (WHO, 2011). Generally, symptoms are mild but can be

fetal in young, elderly or immunocompromised patients. The case fatality rate of *Campylobacter* infection is 0.05 per 1,000 (Smith and Blaser, 1985; Skirrow et al., 1993). Serious complications such as bacteremia, hepatitis, cholecystitis, pancreatitis and peritonitis were reported in some cases (Acheson and Allos, 2001). The most important post-infection complications are reactive arthritis and neurological disorders including Guillain-Barré Syndrome (GBS), an acute paralysis of peripheral nervous system, and Miller Fisher Syndrome. GBS is estimated to be found one in every 2,000-5,000 infections. Around 30% of GBS human cases in the US were associated with *Campylobacter* infection. (Mishu and Blaser, 1993).

Campylobacteriosis is considered a zoonotic disease. The main route of transmission is believed to be foodborne, via consumption of undercooked meat, meat products and offal of poultry, cattle, swine and etc. Consumption of pasteurized milk and contaminated water is also the source of infection (Kaakoush et al., 2015). Additionally, consumption of cooked food cross-contaminated with raw meat juice via kitchen tools has been reported as important mode of transmission (Humphrey et al., 2001; Luber et al., 2006). Numerous outbreaks of food-borne illness associated with *Campylobacter* were reported in the US (CDC, 2015b).

In recent years, human *Campylobacter* infection cases were highly reported in many countries especially in the United States, the United Kingdom, and member states of the European Union. Currently, *Campylobacter* is considered the most common cause of human gastroenteritis in the EU (EFSA, 2013). Likewise, campylobacteriosis is recognized as one of the most frequently reported foodborne illnesses in the US, approximately 2.5 million people are infected each year via consumption of foods contaminating with *Campylobacter* (Mead, 2000). Centers for Disease Control and Prevention (CDC) reported 14.3 *Campylobacter* infection cases

per 100,000 population in the US in 2012 and this incidence was 14 percent higher than that in 2006-8 (CDC, 2013), while in 2014, the incidence of *Campylobacter* was approximately 13 percent higher than in 2006-2008 (CDC, 2015a). In the EU, the average incidence rate of *Campylobacter* reported cases also increased from 43.9 per 100,000 population in 2008 to 48.6 per 100,000 population in 2010 (EFSA, 2011; EFSA, 2013). However, the report incidence rate of *Campylobacter* is thought to be underestimated. CDC reveal that for every *Campylobacter* case reported, there were approximately 30 cases not being diagnosed (CDC, 2014).

2.3 Pathogenicity of *Campylobacter*

More than 90% of *Campylobacter* infections in humans are caused by *C. jejuni* and *C. coli* (WHO, 2011). The infectious dose for *Campylobacter* in humans can be as low as a few hundred cells (Black et al., 1988; Chen et al., 2006; Gormley et al., 2008). Factors involved in the pathogenicity of *Campylobacter* are chemotaxis, binding and adherence, invasion and toxin production (Fauchere et al., 1986). *Campylobacter* has the polar flagellum that helps attach to intestinal epithelial cells and facilitate the colonization in the intestine. Adhesion and binding factors of *C. jejuni* include fibronectin-binding outer membrane protein so called CadF (Moser et al., 1997). *Campylobacter* can invade to the cell after the early mucosal damage by using capsular polysaccharide, sialylation of the lipooligosaccharides and *Campylobacter* invasive antigens (Cia) (Rivera-Amill and Konkel, 1999; Karlyshev and Wren, 2001). Moreover flagellin protein, FlaC, also plays an important role for host cell invasion (Song et al., 2004). Additionally, *Campylobacter* can produce cytolethal discending toxins (CDT) types A, B and C which can cause bloody diarrhea in humans. CDT also plays a role in the invasion and modulation of the immune response by causing the production of interleukin-8 in human which leads to the inflammation to

the intestine (MacCallum et al., 2006; Borrmann et al., 2007). CDT induces the production of neutralizing antibodies in human but not in chicken; therefore, the response to *Campylobacter* infection is different in different kinds of host species (Fauchere et al., 1986; Young et al., 2007).

2.4 Food safety-related aspects of *Campylobacter*

Previous studies revealed that the prevalence of *Campylobacter* contamination was highest in poultry meat compared to other types of meat (Zhao et al., 2001; Wong et al., 2007). Moreover, *Campylobacter* isolates from human cases were highly related to chicken isolates (Mullner et al., 2010). Poultry carcasses were frequently contaminated with this organism and were likely responsible for approximately 70% of sporadic campylobacteriosis cases (Skirrow, 1991; Friedman et al., 2000). Thus, chicken meat was considered a major source of *Campylobacter* infection in humans (Baker et al., 2006; Sheppard et al., 2009b). Of all *Campylobacter* species that can cause disease in humans, *Campylobacter jejuni* is the most common organism found in chickens, followed by *Campylobacter coli* (Moore et al., 2002). The important routes of *Campylobacter* transmission to humans are consumption of undercooked poultry meat and cross-contamination of this organism to cooked meat products (Humphrey et al., 2001; Luber et al., 2006; WHO, 2011). Since little amount of *Campylobacter* ingested (approximately 500-800 cells) can affect human health (Black et al., 1988), the contamination level of *Campylobacter* on chicken carcasses should be concerned.

It was shown that if the number of *Campylobacter* contamination on chicken is less than 1,000 CFU/g of neck and breast skin, the risk of human campylobacteriosis would decrease 50 %. Moreover, if the number of *Campylobacter* contamination on chicken is less than 500 CFU/g of neck and breast

skin, the risk of human campylobacteriosis would decrease more than 90 % (EFSA, 2011).

2.5 *Campylobacter* contamination in poultry slaughterhouse

Many developed countries showed varying trend towards the prevalence of *Campylobacter* on chicken carcasses during processing. In the US, the prevalence of *Campylobacter* on post-chill chicken carcasses ranged from 26-88% (Stern and Pretanik, 2006; Son et al., 2007; Simmons et al., 2008; Richardson et al., 2009; Hue et al., 2010; Schroeder, 2012), while in the UK, 86% of post-chill carcasses were *Campylobacter* positive (Powell et al., 2012). As for *Campylobacter* prevalence on chicken carcasses in the member states of EU, the prevalence widely varied from 4.9-100% (EFSA, 2010). In Thailand, the prevalence of *Campylobacter* on post-chill carcasses ranged from 13.3-100% (Osiriphun et al., 2011; Chokboonmongkol et al., 2013). Not only the prevalence of *Campylobacter* should be considered, but the amount of *Campylobacter* on chicken carcass is also important. The higher the number of *Campylobacter* on chicken carcass, the more likely the people will be affected by this foodborne pathogen. Stern and Pretanik (2006) reported that 3.6% of US broiler carcasses sampled yielded the concentration of *Campylobacter* more than 10^5 CFU/carcass. In the EU, 0-31.9% of the carcasses contained *Campylobacter* more than 10^4 CFU/g of neck skin (EFSA, 2010), while 27% of the carcasses in the UK contained more than 10^3 CFU/g (Powell et al., 2012).

During slaughtering, chicken passes through many processes beginning from stunning and bleeding, followed by scalding, defeathering, evisceration, inside-outside washing and air or water immersion chilling. After that, it may be packed for sale as a whole carcass or further cut and deboned. Carcass contamination can occur at different steps during slaughtering including defeathering, evisceration and

immersion chilling (Figueroa et al., 2009; Guerin et al., 2010; Osiriphun et al., 2011). At slaughterhouse, the number of *Campylobacter* in live chickens varied from 5 to 8 log CFU/g of cecal content (Stern and Robach, 2003; Berrang et al., 2004b; Northcutt et al., 2005; Rosenquist et al., 2006; Hue et al., 2010). If chicken enters the processing plant with such high levels of *Campylobacter*, cross-contamination during slaughtering will be subsequently high. Rosenquist et al. (2006) revealed that the level of *Campylobacter* on fully processed carcasses was directly related to the number of *Campylobacter* in ceca.

Generally, many processing steps, particularly scalding, defeathering and immersion chilling can affect the number of *Campylobacter* on chicken carcasses. Several studies showed 1-3 log reduction of the number of *Campylobacter* on carcass after scalding (Berrang et al., 2000; Northcutt et al., 2005) and the prevalence of *Campylobacter* reduced 20-40% as a result of high temperature of scalding water (approximately 55-60 °C) (Berrang et al., 2000; Vashin and Stoyanchev, 2004). After defeathering, approximately 16-70% increasing in the prevalence of *Campylobacter* and 2 log increasing of *Campylobacter* concentration were reported (Berrang et al., 2000; Vashin and Stoyanchev, 2004). However, Figueroa et al. (2009) reported 6-22% reduction of *Campylobacter* positive samples after defeathering. After evisceration, the number and prevalence of *Campylobacter* on chicken carcass typically increased because of the leakage of intestinal content to the carcass. The percentage of *Campylobacter* positive carcasses after evisceration ranged from 50-100% and the number of *Campylobacter* on carcass ranged from 2.4-3.9 log CFU/g (Corry and Atabay, 2001; Rosenquist et al., 2006; Zhang et al., 2013). Several studies reported a significant increase in *Campylobacter* concentration of 0.5 log CFU/g after evisceration (Corry and Atabay, 2001; Rosenquist et al., 2006).

Inside-outside carcass washing has been introduced to the slaughtering process to reduce fecal contamination from the evisceration before chickens enter the chilling system. Although several studies found 1 log reduction of *Campylobacter* concentration after washing and *Campylobacter* positive carcasses declined 3-23% (Berrang et al., 2000; Oyarzabal et al., 2004; McCrea et al., 2006), other studies did not notice the reduction of *Campylobacter* after washing (Oyarzabal et al., 2004; Son et al., 2007). Changes in the level of *Campylobacter* on post-chill carcasses differ among studies. Many studies reported 10-48% reduction in the prevalence of *Campylobacter* on carcasses after chilling with 0.4-2.2 log reduction in the number of *Campylobacter* (Stern and Robach, 2003; Rosenquist et al., 2006; Allen et al., 2007; Berrang et al., 2007; Son et al., 2007; Figueroa et al., 2009). However, some studies did not find the decrease in number and prevalence of *Campylobacter* after chilling (Reiter et al., 2005; Jozwiak et al., 2006).

2.6 Method for *Campylobacter* recovery and enumeration

The contamination of *Campylobacter* on chicken carcass during slaughter is mostly originated from feces and intestinal content. These microorganisms were likely trapped within biofilm on the surface of chicken carcass during processing (Buswell et al., 1998; Rahimi et al., 2010). *Campylobacter* can persist in the skin of live poultry and lead to carcass contamination during slaughter (Franchin et al., 2005).

Carcass rinse sampling technique with buffer peptone water has been recognized as the standard method for foodborne pathogens including *Campylobacter* detection by the US Department of Agriculture Food Safety and Inspection Service (USDA, 2013). This technique is able to recover microorganisms that are trapped outside and inside surface of the carcass. Thus, it has been widely

used for the detection of *Campylobacter* on chicken carcasses (Cox et al., 2010; Zhang et al., 2013). Jorgensen et al. (2002) revealed that the likelihood of isolating *Campylobacter* from neck skin, carcass rinse, carcass rinse plus whole skin samples was similar.

Direct plating method has been used for detection and enumeration of *Campylobacter* on chicken carcasses in many countries especially in the US and the UK because it is rapid, simple and cost effective (Line et al., 2001; Oyarzabal et al., 2005; Scherer et al., 2006). Direct plating method also allows the recovery of multiple *Campylobacter* subtypes (Thomas et al., 1997; Newell and Fearnley, 2003; Oyarzabal et al., 2005). However, several studies found that the prevalence of *Campylobacter* was underestimated when it was determined by the direct plating method (Stern and Pretanik, 2006; Berrang et al., 2007; Richardson et al., 2009). Selective enrichment method, on the other hand, is considered to be a better method for recovery of *Campylobacter* from post-chill carcasses. Nutrient broth enhances the recovery of sublethally injured cells as *Campylobacter* is sensitive to environmental stress (Shimada and Tsuji, 1986; Humphrey, 1989; Richardson et al., 2009). Double strength nutrient broth was used to reduce the volume of nutrient broth adding to sample. According to the Advisory Committee on the Microbiological Safety of Food (ACMSF) Surveillance Working Group (2010) and several studies (Humphrey, 1994; Jorgensen et al., 2002; Allen et al., 2007), selective enrichment method with Exeter broth provided good results for *Campylobacter* recovery from different types of sample including chicken carcass rinse. Exeter broth has also been adopted by the UK Department of Health to use for monitoring *Campylobacter* in food samples (Humphrey, 1994). Despite selective enrichment broth suppresses the growth of non-*Campylobacter* organisms (Baylis et al., 2000; Richardson et al., 2009),

this method could cause the selection bias on subtypes of *Campylobacter* isolated as it promotes the growth of certain *Campylobacter* strains (Williams et al., 2012). Thus, in order to increase the accuracy of *Campylobacter* prevalence recovered from chicken carcasses, the combination of direct plating and selective enrichment methods should be used (Habib et al., 2008; Figueroa et al., 2009; Richardson et al., 2009).

2.7 Genotyping techniques and genetic characteristics of *Campylobacter* spp.

Many genotyping techniques have been used to determine genetic relatedness of *Campylobacter* isolates in poultry industry. Sequence-based typing method is commonly used for studying the genetic of foodborne bacterial pathogens including *Campylobacter* (Clarke, 2002; Patchanee et al., 2012; Cody et al., 2013). The nucleotide sequencing of the *flaA*-short variable region (*flaA*-SVR) has been developed for genotyping and it provided similar results to that of the whole *flaA* gene (Meinersmann et al., 1997). The *flaA*-SVR sequencing has been commonly used to determine genetic relatedness among *Campylobacter* isolates because it provided high discriminatory power (Dingle et al., 2005; Hiett et al., 2006; Hunter et al., 2009; Wassenaar et al., 2009; Perko-Makela et al., 2011). Similar to *flaA*-SVR sequencing, multilocus sequence typing (MLST) is another sequence-based typing method that is commonly used for studying the genetic of foodborne pathogens (Dingle et al., 2005; Patchanee et al., 2012). With the high discriminatory power and online database availability, MLST is a suitable tool for molecular characterization of *Campylobacter jejuni* (Mullner et al., 2010). Many studies have been successful with using *flaA*-SVR sequencing and MLST for genetic characterization as they provided results equivalent to PFGE (Manning et al., 2003; Sails et al., 2003; Dingle et al., 2005).

Despite *Campylobacter* contamination on chicken carcasses is usually originated from fecal contamination, *Campylobacter* isolates recovered from fully processed carcasses might not have the same subtypes as the isolates from cloacal swabs or ceca (Lindmark et al., 2006). Several studies in the US, the EU and the UK found that chicken carcasses in slaughterhouses were contaminated with 1-3 *Campylobacter* subtypes (Dickins et al., 2002; Lindmark et al., 2006; Hunter et al., 2009). In addition, some studies showed that number of *Campylobacter* subtypes decreased as chickens passed through the chilling process and only certain subtypes could be recovered from fully processed carcasses (Newell et al., 2001; Hiett et al., 2002; Berrang et al., 2007; Hunter et al., 2009).

2.8 Studies of *Campylobacter* in Thailand

Unlike the European countries or the US, campylobacteriosis has been rarely reported in Thailand. Among foreigners who travelled to Thailand, *Campylobacter* is one of the most common pathogens that causes diarrhea (Kuschner et al., 1995; Sanders et al., 2002; Serichantalergs et al., 2010; CDC, 2015c). Diarrhea caused by *Campylobacter* was mainly reported in Thai children at the age between 2 - 12 years old (Taylor et al., 1991; Acheson and Allos, 2001; Bodhidatta et al., 2002). Additionally, Padungtod and Kaneene (2005) found that 18% of children with diarrhea in northern part of Thailand were infected with *Campylobacter*, while 5% of farm workers in that study were positive for *Campylobacter* and no *Campylobacter* was isolated from healthy non-farm residents. Despite the under reported prevalence of *Campylobacter* in human, chicken meat products in Thailand were highly contaminated with *Campylobacter*. The contamination rate of Thai retailed chicken products was 52-90.63% (Meeyam et al., 2004; Vindigni et al., 2007; Suzuki and Yamamoto, 2009).

In Thailand, 20-100% of chicken carcasses during slaughtering process were contaminated with *Campylobacter* (Padungtod and Kaneene, 2005; Osiriphun et al., 2011; Chokboonmongkol et al., 2013). High level of contamination at plucking step was reported (Osiriphun et al., 2011). The concentration of *Campylobacter* on chicken carcasses increased after defeathering (2.98 log CFU) and after evisceration (2.88 log CFU). The contamination level on carcasses decreased to 0.85 log CFU after chilling (Osiriphun et al., 2011). Additionally, *Campylobacter* genotypes obtained from carcasses in Thai slaughterhouse were related to the isolates from the broiler flocks (Chokboonmongkol et al., 2013).

CHAPTER III

MATERIALS AND METHODS

3.1 Sample collection

Samples were collected from broiler flocks of *Campylobacter*-positive farms slaughtered at 3 slaughterhouses during June 2012 to April 2013. In total, 40 cloacal swabs, 200 chicken carcass rinses and 80 ceca were obtained from 8 broiler flocks. For each flock, 5 clocal swabs, 25 carcass rinses and 10 ceca were collected. During the slaughtering process, samples were taken from 5 steps including scalding, defeathering, evisceration, inside-outside washing and immersion chilling (Figure 1). Five carcasses were collected from each step. Chicken carcass rinsing was performed according to USDA (2013). Briefly, each carcass was placed into sterile bag containing 400 ml of buffered peptone water (BPW) and was vigorously shaken for 1 min. Carcass rinses were kept in closed container and placed on ice and brought back to the laboratory within 4 h.

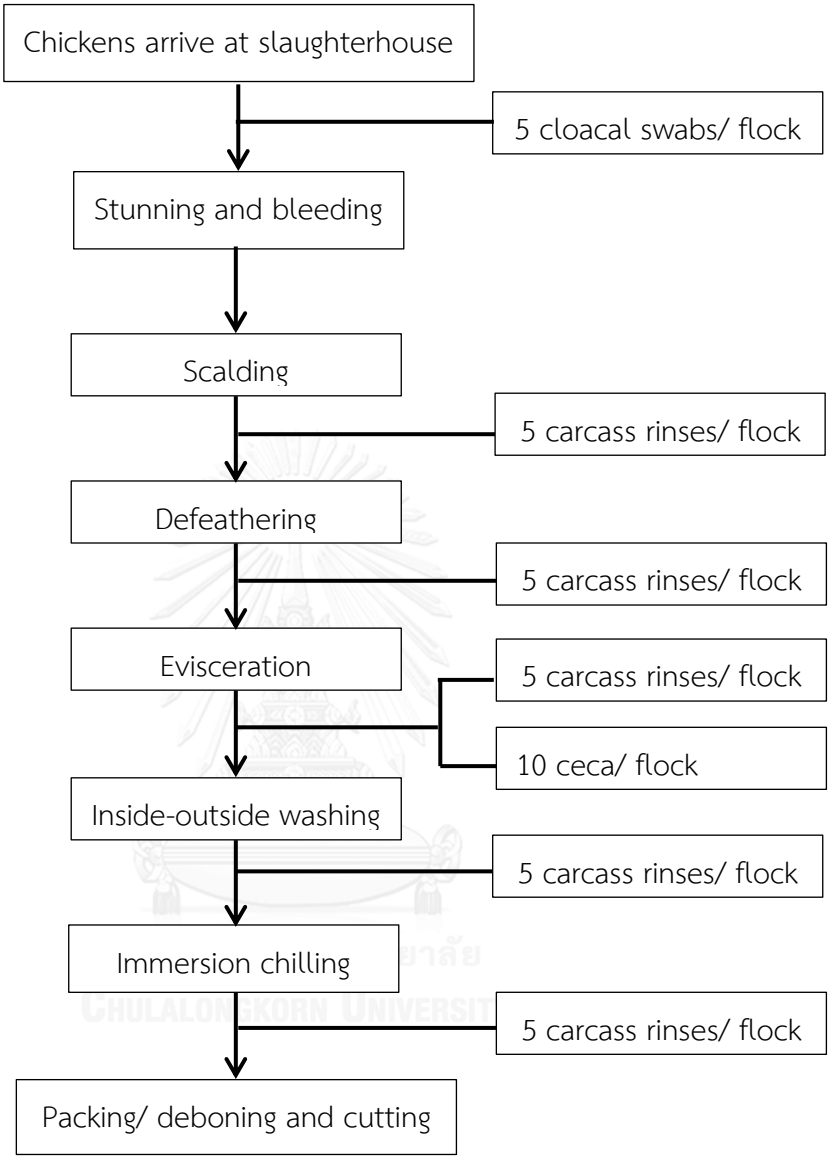

Figure 1. Sampling procedures of this study (per flock)

3.2 *Campylobacter* isolation and enumeration

3.2.1 Carcass rinse

Direct plating method was used for detection and enumeration of *Campylobacter* on chicken carcasses. Methods were modified from the International Organization for Standardization (ISO, 2006b). Briefly, 100 µl of carcass rinse were directly spread onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA). Then, the inoculated plate was incubated at 42 °C for 48 h under a microaerobic condition (85% N2, 10% O2 and 5% CO2) (ISO, 2006a).

In addition, selective enrichment method with Exeter broth and double-strength Exeter broth was used for enhancing the recovery of *Campylobacter* from chicken carcass rinse. To perform selective enrichment method with Exeter broth, 1 ml of carcass rinse was added to 9 ml of Exeter broth containing selective supplements (Appendix A) and then gently mixed. The method was modified from ISO (ISO, 2006b). The mixture was incubated at 37 °C for 48 h under a microaerobic condition. Thereafter, 100 µl of enriched broth was spread on mCCDA in duplication. The inoculated plate was incubated at 42 °C for 48 h as previous described. For selective enrichment method with double-strength Exeter broth, 25 ml of carcass rinse were added to 25 ml of double strength Exeter nutrient broth (Humphrey, 1994) and then mixed thoroughly. The method was modified by USDA (USDA FSIS, 2013). The mixture was incubated at 37 °C for 48 hours under a microaerobic condition. After that, enriched sample was seeded on mCCDA as similar to previously described for selective enrichment with Exeter broth.

The typical morphology of *Campylobacter* colonies were metallic greyish, flat, and thinly spread. Presumptive colonies were selected and purified by subculturing onto blood agar. Each *Campylobacter* isolate was further kept in a

cryovial tube containing skim milk and 30% (v/v) glycerol and stored at -80℃ for further studies.

In terms of *Campylobacter* enumeration, 1 ml of carcass rinse was diluted with sterile normal saline to make a ten-fold dilution. One hundred microliters of 10^{-2} suspension were spread on duplicate mCCDA plates and then incubated at 42 ℃ for 48 h under a microaerobic condition. After incubation, presumptive *Campylobacter* colonies were counted and concentration of *Campylobacter* on carcass in CFU/ml was calculated.

3.2.2 Cecum

To detect *Campylobacter* in cecum, cecal content was directly steak onto mCCDA plate. Inoculated plates were incubated at 42 ℃ for 48 h under a microaerobic condition (Hook et al., 2005).

For the enumeration of *Campylobacter* in cecum, 1 g of cecal content from each cecum was diluted with 9 ml of normal saline to make a ten-fold dilution. One hundred microliters of the 10^{-3} and 10^{-5} dilution were plated onto mCCDA in duplication and then incubated under a microaerobic condition. Number of presumptive *Campylobacter* colonies on each plate was counted after 48 h of incubation and concentration of *Campylobacter* in CFU/g of cecal content was calculated.

3.2.3 Cloacal swab

For *Campylobacter* isolation from cloacal swab, cloacal swabs were directly streaked onto mCCDA and incubated at 42 ℃ for 48 h under a microaerobic condition as mentioned above.

3.3 *Campylobacter* confirmation and species identification

Presumptive *Campylobacter* colonies were confirmed by multiplex PCR according to the previous publications by Linton et al. (1996) and Wang et al. (2002). DNA extraction was performed by conventional boiling method. Confirmation of *Campylobacter* genus was performed with primers specific for *Campylobacter* 16S rRNA gene. The PCR condition was composed of denaturation at 94 ℃ for 12 min, followed by 30 cycles of 94 ℃ for 30 sec, 55 ℃ for 60 sec and 72 ℃ for 60 sec. The size of PCR product was 816 bp (Linton et al., 1996). To identify *Campylobacter* species, oligonucleotide primers which hybridize to *hipO* gene of *C. jejuni* and *glyA* gene of *C. coli* were used as previously described by Wang et al. (2002). Primers were shown in Table 1. The PCR condition for species identification comprised of denaturation at 95 ℃ for 15 min, followed by 30 cycles of 94 ℃ for 20 sec, 55 ℃ for 20 sec and 72 ℃ for 30 sec and then a final extension step at of 72 ℃ for 5 min (Wang et al., 2002). PCR products were analyzed by electrophoresis in 1.2 % (w/v) agarose gels stained with ethidium bromide and visualized in a UV gel document system. A 323-bp amplicon was generated for *C. jejuni* and a 126-bp amplicon was generated for *C. coli*.

3.4 Genetic characterization of *Campylobacter* isolates

In order to determine the genetic relatedness of *Campylobacter* strains isolated from chicken carcass during slaughtering process, *flaA*-short variable region (*flaA*-SVR) sequencing was performed as previously described by Meinersmann et al. (1997). A 35 cycle-reaction consisted of 30 sec denaturing at 96 ℃, 45 sec annealing at 52 ℃ and 1 min extension at 72 ℃ (Meinersmann et al., 1997). The 425-bp PCR product was cleaned using PCR clean-up kit and sent for sequencing at First BASE Laboratories, Malaysia. Nucleotide sequences were submitted to *flaA*-SVR database to identify *flaA* allele number at http://pubmlst.org/*Campylobacter*/*flaA*.

3.5 Statistical analysis

Statistical analysis was carried out using SPSS software version 22.0 (IBM, New York, USA). Chi-square was used to compare the difference in the occurrence among different slaughtering processes and different flocks. ANOVA was used to compare the difference in *Campylobacter* concentration among different flocks and different slaughtering processes. A *p*-value of < 0.05 was considered as significant difference.

Table 1. Oligonucleotide primers used in this study

Function	Target gene	Primer sequences 5' -3'		Amplicon size (bp)	Reference
		Forward primer	Reverse primer		
Genus identification	16S rRNA	GGATGACACTTTTCGGAGC	CATTGTAGCACGTGTGTC	816	Linton et al., 1996
Species identification					
C. jejuni	*hipO*	ACTTCTTTATTGCTTGCTGC	GCCACAACAAGTAAAGAAGC	323	Wang et al., 2002
C. coli	*glyA*	GTAAAACCAAAGCTTATCGTG	TCCAGCAATGTGTGCAATG	126	
flaA-SVR sequencing	*flaA*	CTATGGATGAGCAATTWAAAAT	CAA GWCCTGTTCCWACTG AAG	425	Meinersmann et al., 1997

CHAPTER IV

RESULTS

4.1 Occurrence of *Campylobacter* at different slaughtering processes

Occurrence of *Campylobacter* in chicken carcasses collected from 8 broiler flocks from *Campylobacter* positive farms slaughtered at 3 slaughterhouses was shown in Table 2. Of 320 samples included in this study, 233 samples (72.81%) were *Campylobacter* positive. *Campylobacter jejuni* was the major species contaminating chicken carcasses which accounted for 92.01% of the isolates. Only 7.99% of the isolates were *C. coli*. In addition, *C. coli* was found only in one out of 8 broiler flocks (flock C).

During slaughter, *Campylobacter* positive carcasses were mostly found after defeathering (85%, 34 out of 40), after evisceration (82.5%, 33 out of 40) and after inside-outside washing (80%, 32 out of 40). Seventy-five percent (30 out of 40) of carcass rinses were positive for *Campylobacter* after chilling, the final step of slaughtering process. In contrast to other processes, only 50% (20 out of 40) of chicken carcasses obtained after scalding were *Campylobacter* positive. However, the difference in *Campylobacter* occurrence among different slaughtering processes was not statistically significant.

In most of the *Campylobacter* positive broiler flocks, *Campylobacter* was found almost 100% of cloacal swabs and ceca except in some flocks (flocks F and H) (Table 2). When Campylobacter cannot be found in cloacal swabs, no *Campylobacter* was detected in ceca as well (Figure 2). The occurrence of *Campylobacter* in carcass rinse was different among broiler flocks. Among 8 *Campylobacter* positive flocks included in this study, 5 flocks (flock D – H) were

slaughtered as the first flock of the day. The occurrence of *Campylobacter* in carcass rinses collected from these flocks ranged from 16 – 100%. As for flocks A and B which were slaughtered as the forth and third flock of the day, the occurrence of *Campylobacter* was 60% and 80%, respectively. Interestingly, 88% of carcass rinses from flock C which was the second flock slaughtered were *Campylobacter* positive. Overall, the contamination rate of *Campylobacter* in carcass rinse was related to the occurrence of *Campylobacter* in cecum and cloacal swab. When the occurrence of *Campylobacter* in cecum and cloacal swab was high, the occurrence of *Campylobacter* in carcass rinse was also high. On the other hand, when *Campylobacter* was not detected in cloacal swab sample or cecum, the occurrence of *Campylobacter* in carcass rinse was low. However, in flock F, the contamination rate of carcass rinse was high (84%) despite the low occurrence of *Campylobacter* in cecum (20%) and no detection of *Campylobacter* in cloacal swab sample.

Eight *Campylobacter* positive flocks were slaughtered at 3 slaughterhouses. Four flocks (A, B, C and D) were slaughtered at slaughterhouse I. Two flocks (flocks E and F) were slaughtered at slaughterhouse II and the other two flocks (flocks G and H) were slaughtered at slaughterhouse III. When the contamination rate of *Campylobacter* on carcasses in each slaughterhouse was compared, slaughterhouse II had the highest occurrence of *Campylobacter* in carcass rinse (84%, 42 out of 50), followed by slaughterhouse I (78%, 78 out of 100) and slaughterhouse III (58%, 29 out of 50), respectively.

Table 2. Occurrence of *Campylobacter* on chicken carcasses, ceca and cloacal swabs collected from 8 *Campylobacter* positive broiler farms

Plant	Flock	No. of positive samples/ tested samples (%)							
		Cloacal swab	Carcass rinse						Cecal content
			After scalding	After defeathering	After evisceration	After I/O washing	After chilling	Overall	
I	A	5/5 (100)	0/5 (0)	5/5 (100)	3/5 (60)	4/5 (80)	3/5 (60)	15/25 (60)	8/10 (80)
	B	5/5 (100)	2/5 (40)	5/5 (100)	5/5 (100)	3/5 (60)	5/5 (100)	20/25 (80)	10/10 (100)
	C	NA	2/5 (40)	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)	22/25 (88)	9/10 (90)
	D	5/5 (100)	4/5 (80)	4/5 (80)	4/5 (80)	5/5 (100)	4/5 (80)	21/25 (84)	10/10 (100)
II	E	5/5 (100)	3/5 (60)	5/5 (100)	5/5 (100)	4/5 (80)	4/5 (80)	21/25 (84)	10/10 (100)
	F	0/5 (0)	3/5 (60)	5/5 (100)	4/5 (80)	5/5 (100)	4/5 (80)	21/25 (84)	2/10 (20)
III	G	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)	25/25 (100)	10/10 (100)
	H	0/5 (0)	1/5 (20)	0/5 (0)	2/5 (40)	1/5 (20)	0/5 (0)	4/25 (16)	0/10 (0)
	Total	25/40 (62.5)	20/40 (50)	34/40 (85)	33/40 (82.5)	32/40 (80)	30/40 (75)	149/200 (74.5)	59/80 (73.6)

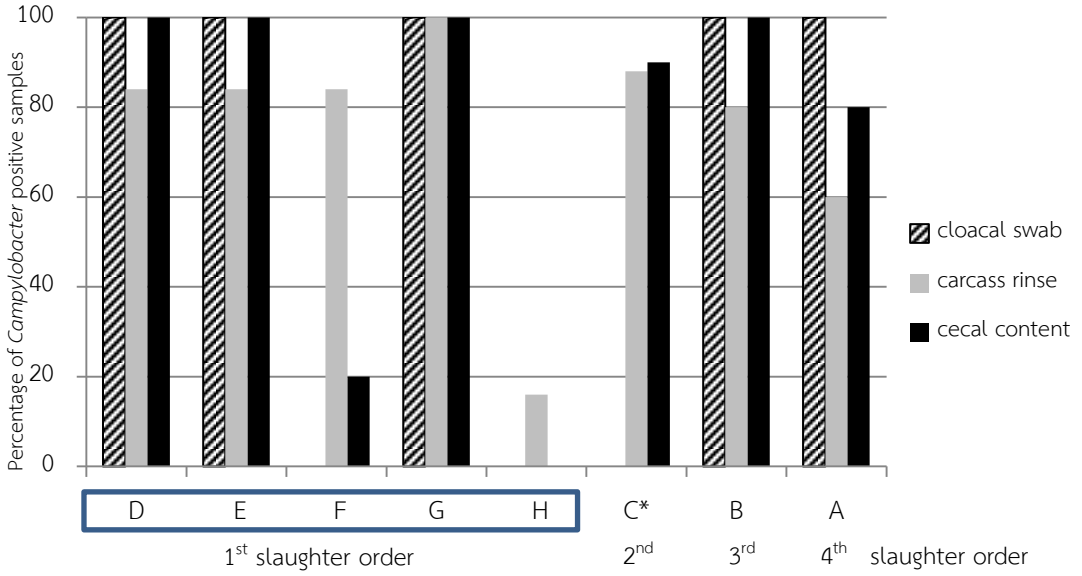

Figure 2. Occurrence of *Campylobacter* in different sample types collected from 8 *Campylobacter* positive farms

* No cloacal swabs were collected in flock C

4.2 Concentration of *Campylobacter* in chicken carcasses and ceca

Mean concentrations of *Campylobacter* on chicken carcasses during slaughter were summarized in Table 3. Overall, the number of *Campylobacter* significantly increased after defeathering (p = 0.01). The mean concentration was 2.76 ± 0.16 (concentration ranged from 1.08 to 3.66 log CFU/ml) and 1.88 ± 0.30 (concentration ranged from 1.00 to 3.12 log CFU/ml) after defeathering and scalding, respectively. Later, after evisceration, the mean concentration of *Campylobacter* increased significantly (p = 0.04) to 3.26 ± 0.15 (2.28 - 3.90 log CFU/ml). In most broiler flocks, the number of *Campylobacter* increased after evisceration, except in flock E which the concentration of *Campylobacter* decreased after evisceration step. Number of *Campylobacter* remained high after inside-outside washing (p = 0.60) which mean concentration was found at 3.42 ± 0.13 (3.29 - 4.00 log CFU/ml). Finally, after chilling, the number of *Campylobacter* dropped significantly (p = 0.001) to 2.04

± 0.27 log CFU/ml. The level of *Campylobacter* contamination on chicken carcasses after chilling step decreased in every flock except in flock C which the high number of *Campylobacter* was still detected. The concentration of *Campylobacter* after chilling broadly ranged from 1.09 to 4.00 log CFU/ml.

Campylobacter concentration on chicken carcasses throughout the slaughtering process was different among broiler flocks (Figures 3) and slaughterhouses (Table 4). When the concentration of *Campylobacter* in each slaughterhouse was compared, the highest contamination level of *Campylobacter* in carcass rinses obtained during slaughter (2.98 ± 0.31 log CFU/ml) was noticed in slaughterhouse II, followed by slaughterhouse I (2.66 ± 0.36 log CFU/ml) and slaughterhouse III (2.32 ± 0.49 log CFU/ml), respectively (Table 4).

Mean *Campylobacter* concentration in cecum in this study was 7.33 ± 0.49 log CFU/g of cecal content. *Campylobacter* concentration in cecum in each flock ranged from 5.45 – 9.02 log CFU/g of cecal content. In flock D, *Campylobacter* concentration in cecum was lowest (5.45 log CFU/g), while in flock C, the concentration in cecum was highest (9.02 log CFU/g). The results were shown in Table 4. In each broiler flock, *Campylobacter* concentration in cecum was approximately 3-5 log higher than that in carcass rinse.

Table 3. Concentration of *Campylobacter* in carcass rinse and cecal content

Plant	Flock	Mean *Campylobacter* concentration in carcass rinse (log CFU/ml± SE)						Cecal content
		After scalding	After defeathering	After evisceration	After I/O washing	After chilling	Overall	(CFU/g ± SE)
I	A	-	2.94 ± 0.14	3.90**	3.93 ± 0.07	2.84 ± 0.22	3.36 ± 0.30	8.61 ± 0.23
	B	1.30 ± 0.35	2.07 ± 0.30	2.28 ± 0.53	3.29**	1.96 ± 0.28	2.09 ± 0.32	5.85 ± 0.60
	C	2.13 ± 0.16	3.50 ±0.44	3.62 ± 0.35	4.00**	4.00 ± 0.00	3.37 ± 0.34	9.02 ± 0.19
	D*	1.00**	1.80 ± 0.39	3.46 ± 0.26	3.32 ± 0.29	-	2.13 ± 0.60	5.45 ± 0.54
II	E	3.12 ± 0.71	3.66 ± 0.15	3.39 ± 0.20	3.29 ± 0.36	1.86 ± 0.29	2.98 ± 0.31	7.85 ± 0.12
	F*	-	-	-	-	-	-	-
III	G	1.69 ± 0.34	3.01 ± 0.18	3.57 ± 0.21	3.38 ± 0.17	1.09 ± 0.10	2.32 ± 0.49	7.97 ± 0.20
	H*	-	-	-	-	-	-	-
Overall		1.88 ± 0.30[a]	2.76 ± 0.16[b]	3.26 ± 0.15[c]	3.42 ± 0.13[c]	2.04 ± 0.27[a]	2.65 ± 0.25	7.33 ± 0.49

* No result on the enumeration in flocks D (after chilling), F and H as the samples were negative for *Campylobacter* by direct plating method.

** Only one sample was positive after enumeration; therefore, no SE was shown.

Different letter (a, b, c and d) within each row refer to the statistically significant difference of *Campylobacter* concentration among slaughtering process

Table 4. Concentration of *Campylobacter* on chicken carcasses in 3 slaughterhouses

Slaughterhouse	Flock	Mean *Campylobacter* concentration (log CFU/ml) ± SE					
		After scalding	After defeathering	After evisceration	After I/O washing	After chilling	Overall
I	A, B, C, D	1.40 ± 0.29	2.49 ± 0.39	3.25 ± 0.36	3.62 ± 0.19	2.81 ± 0.51	2.66 ± 0.36
II	E, F*	3.12 ± 0.71	3.66 ± 0.15	3.39 ± 0.20	3.29 ± 0.36	1.86 ± 0.29	2.98 ± 0.31
III	G, H*	1.69 ± 0.34	3.01 ± 0.18	3.57 ± 0.21	3.38 ± 0.17	1.09 ± 0.10	2.32 ± 0.49

* The number of *Campylobacter* in these flocks were not calculated because none of the samples were positive by direct plating method.

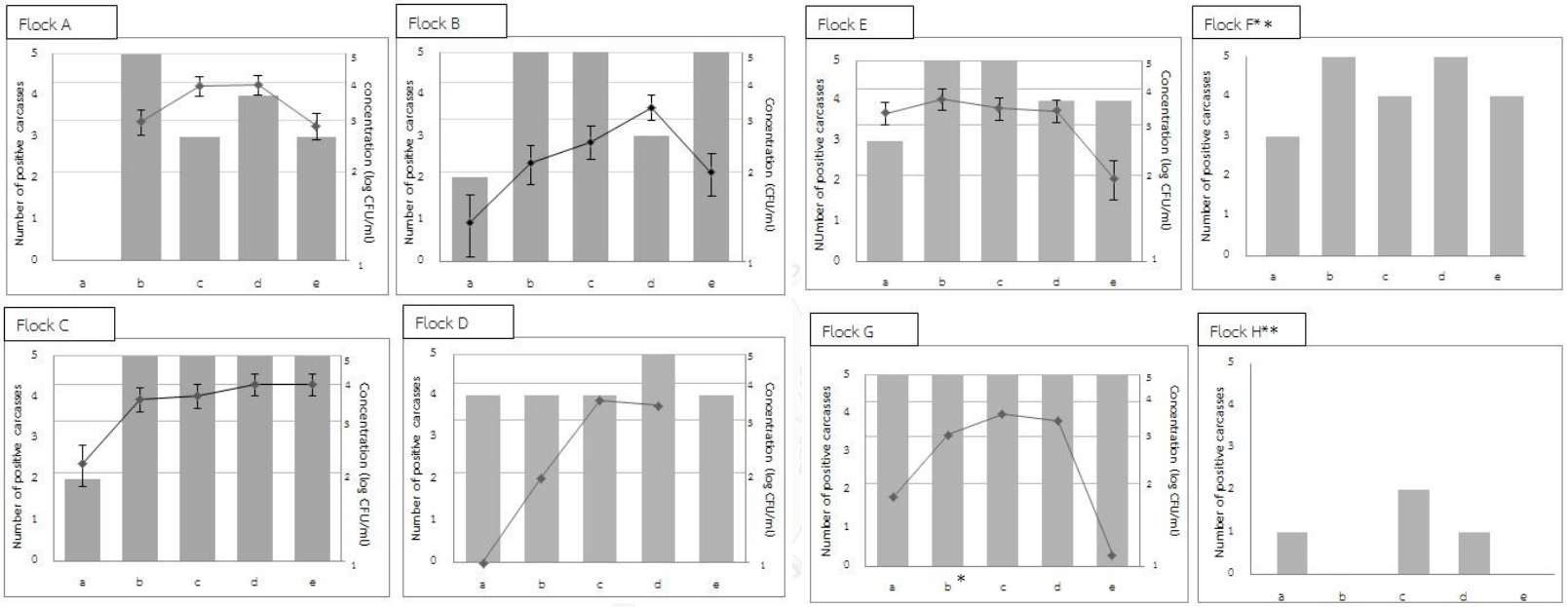

Figure 3. Occurrence and concentration of *Campylobacter* on chicken carcass after scalding, defeathering, evisceration, inside-outside washing and immersion chilling

Line graph indicates concentration of *Campylobacter* and bar graph illustrates occurrence of *Campylobacter* in 5 carcasses from each step.

a = after scalding; b = after defeathering; c = after evisceration; d = after I/O washing; e = after chilling

* In flock D, the number of *Campylobacter* cannot be detected after chilling. *Campylobacter* positive carcass was detected by selective enrichment method because of the sub-lethally injured cells.

**In flock F and flock H, numbers of *Campylobacter* positive carcass were obtained from selective enrichment method. Carcass rinses were *Campylobacter* negative by direct plating method; therefore, there were no enumeration results.

4.3 Genetic profiles of *Campylobacter* isolates from broilers throughout slaughtering process

To determine genetic profiles of *Campylobacter* isolates recovered from broilers during slaughtering process, 304 *Campylobacter* isolates from carcass rinses, ceca and cloacal swabs were genotyped by *flaA*-SVR sequencing. Twenty-six *flaA*-SVR types were detected from 8 broiler flocks. In this study, *flaA*-SVR allele 208 and 783 were mostly found. Each broiler flock had different pattern of *flaA*-SVR type recovery. Some broiler flocks were contaminated with a single type throughout the slaughtering process, while other flocks were contaminated with multiple genotypes.

Three broiler flocks (flock D, G and H) which were slaughtered as the first flock of the day were contaminated with a single subtype throughout the slaughtering line (Table 6). *FlaA*-SVR allele types obtained from flock D, G and H were 783, 208 and 97, respectively. Although flocks E and F were also slaughtered as the first flocks, these flocks were contaminated with 7 and 9 subtypes during slaughtering process, respectively. *FlaA*-SVR allele 22 was the predominant genotype found in flock E, while *flaA*-SVR allele 523 was commonly found in flock F. These genotypes were obtained along the slaughtering process from cloacal swabs, ceca to post-chill carcasses. Similar to flocks E and F, flocks A, B and C were also contaminated with multiple subtypes during slaughter. Flock A which was the fourth flock slaughtered, was contaminated with 4 subtypes. *FlaA*-SVR allele 162 was predominant subtype in this flock. Flocks B and C which were the third and second flock slaughtered were contaminated with 9 and 7 subtypes throughout the slaughtering line. Flock B was mainly contaminated with *flaA*-SVR allele 287, whereas *flaA*-SVR alleles 769 and 54 were commonly found in flock C. In general, *flaA*-SVR types obtained from carcass rinse were quite related to *flaA*-SVR types recovered from cecum and cloacal swabs. However, in flock B, *flaA*-SVR allele 287 was mainly

found on carcasses but this allele was not detected in cecal content. Likewise, in flock C, despite *flaA*-SVR allele 54 was commonly found on carcasses, there was no detection of this allele in cecal content. Therefore, when *flaA*-SVR types among carcass, cloacal swab and cecum were compared, *flaA*-SVR types recovered from carcass rinse were more diverse than those recovered from cecum and clocal swab samples (Table 5). *FlaA*-SVR types obtained from carcass rinse during each slaughtering process were similar. However, in flock with multiple subtypes, number of *flaA*-SVR types recovered from carcass rinse increased after defeathering and decreased after chilling (Table 5).

Table 6. Genetic profiles of *Campylobacter* isolates from chickens at different slaughtering processes

Plant	Flock	*flaA*-SVR type (no. of isolates)							
		Cloacal swab	Carcass rinse						Cecal content
			after scalding	after defeathering	after evisceration	after I/O washing	after chilling	overall	
I	A	162 (2)	n/a	783 (5), 162 (4)	783 (2)	783 (4), 162 (1)	783 (5), 581 (1)	783 (16), 162 (5), 581 (1)	162 (3), 783 (3), 1458 (1)
	B	22 (2), 1527 (1)	287 (2), 57 (1), 312 (1)	287 (4), 45 (1), 253 (1), 312 (1), 1527 (1),	45 (4), 287 (3), 356 (1), 652 (1), 1527 (1)	287 (4)	45 (3), 287 (2), 22 (1), 57 (1), 253 (1)	287 (15), 45 (8), 57 (2), 253 (2), 312 (2), 1527 (2), 22 (1), 356 (1), 652 (1)	45 (8)
	C	n/a	54 (1), 769 (1)	769 (4), 54 (3), 1571 (1)	769 (5), 54 (5), 22 (1), 236 (1)	769 (3), 54 (2), 236 (1), 1323 (1)	769 (5), 54 (4)	769 (18), 54 (15), 22 (1), 236 (1), 1323 (1), 1571 (1)	769 (6), 1525 (1)
	D	783 (5)	783 (5)	783 (4)	783 (8)	783 (9)	783 (4)	783 (30)	783 (7)
II	E	22 (2), 18 (2), 57 (1), 312 (1)	22 (2), 57 (1)	22 (3), 312 (2), 18 (1)	22 (4), 18 (1), 57 (1), 312 (1), 1279 (1)	22 (2), 312 (1), 1582 (1)	22 (3), 18 (1)	22 (14), 312 (4), 18 (3), 57 (2), 1279 (1), 1582 (1)	22 (5), 523 (1)
	F	n/a	523 (5)	287 (2), 315 (2), 57 (1), 177 (1), 288 (1), 523 (1)	287 (3), 57 (1), 177 (1), 288 (1), 523 (1), 1388 (1)	287 (4), 523 (4), 177 (1), 1354 (1)	523 (6)	523 (17), 287 (9), 177 (3), 57 (2), 288 (2), 315 (2), 1354 (1), 1388 (1)	177 (1), 1016 (1)
III	G	208 (5)	208 (8)	208 (12)	208 (11)	208 (9)	208 (10)	208 (50)	208 (7)
	H	n/a	97 (1)	n/a	97 (2)	97 (1)	n/a	97 (4)	n/a

CHAPTER V

DISCUSSION

In this study, the occurrence of *Campylobacter* on chicken carcasses at slaughterhouse was higher than those previously reported in Thailand (Padungtod and Kaneene, 2005; Chokboonmongkol et al., 2013). Chokboonmongkol et al. (2013) and Padungthod and Kaneene (2005) revealed that the prevalence of *Campylobacter* in chicken at slaughterhouse was 51% and 38%, respectively. Although the difference in the occurrence of *Campylobacter* between our study and other studies in Thailand was observed, it should be noted that *Campylobacter* was isolated from broiler skin in previous studies, whereas in this study, *Campylobacter* was isolated from chicken carcass rinse. In addition, the difference in the occurrence of *Campylobacter* in chicken during slaughter might be affected by *Campylobacter* status in the chicken flock prior to slaughter as this study was focused on the *Campylobacter* positive flocks slaughtered.

After defeathering process, a 0.53-2.16 log increasing in number of *Campylobacter* on carcasses and 40-100% increasing in the occurrence were noticed. These findings were similar to previous studies which found 2 log increasing in *Campylobacter* concentration and approximately 16-70% increasing in the prevalence of *Campylobacter* on chicken carcasses after defeathering step (Berrang et al., 2000; Vashin and Stoyanchev, 2004). Increasing in *Campylobacter* contamination was likely due to the leakage of intestinal content through the vent and the contamination of *Campylobacter* from feather and skin to carcass (Chantarapanont et al., 2003; Davis and Conner, 2007; Sampers et al., 2008; Berrang et al., 2011). Although most flocks in the present study had higher *Campylobacter*

number after defeathering, there was 0.02-0.75 log reduction in two flocks (flocks B and E).

After evisceration, the mean *Campylobacter* concentration in most broiler flocks increased. Concentration increased 0.12-1.66 log and the occurrence remained at high level as previous step when comparing the change in the level of *Campylobacter* concentration in each flock. Several studies also did not find the change in occurrence between after defeathering and evisceration (54.8% vs 51.2% and 68% vs 69.4%) (Franchin et al., 2007; Rahimi et al., 2010). As for the concentration of *Campylobacter* after evisceration step compared to defeathering step, approximately 0.5 log increasing in carcasses after evisceration was reported (Corry and Atabay, 2001; Rosenquist et al., 2006). An increase in *Campylobacter* concentration might be due to the rupture of intestinal tract, leading to the contamination of *Campylobacter* to carcasses.

Inside-outside carcass washing has been introduced to the slaughtering process to reduce fecal contamination on chicken carcasses from previous steps. However, in this study, the contamination rate and the concentration after I/O washing was still high in most flocks. Only small change in the occurrence and concentration after I/O washing was notified. Some broiler flocks had 0.03-0.3 log increase in concentration, while others had 0.1-0.2 log reduction. Likewise, several studies did not find the reduction of *Campylobacter* after washing (Oyarzabal et al., 2004; Jozwiak et al., 2006; Son et al., 2007), except in 2 particular studies which reported 1 log reduction of *Campylobacter* concentration after washing and 3-23% reduction in *Campylobacter* contamination rate (Berrang et al., 2000; McCrea et al., 2006).

Although only a minimal change was observed after washing step, a drastically reduction in the level of *Campylobacter* was noticed after chilling. Approximately 1-2 log reduction in each flock was observed in this study. Previous studies also found 0.4-2.2 log reduction in the concentration of *Campylobacter* after chilling step (Stern and Robach, 2003; Rosenquist et al., 2006; Berrang et al., 2007; Son et al., 2007; VM et al., 2007; Figueroa et al., 2009). Although the great change in the concentration in our study was observed, the concentration of *Campylobacter* on post-chill carcasses was still high compared to previous studies (Berrang and Dickens, 2002; Berrang et al., 2007) because chlorination were generally used in the chilling tank in previous studies.

Although several studies reported 10-48% reduction in the prevalence of *Campylobacter* on post-chill carcasses (Stern and Robach, 2003; Rosenquist et al., 2006; Berrang et al., 2007; Son et al., 2007; VM et al., 2007; Figueroa et al., 2009), our study did not noticed greatly changed in the number of *Campylobacter* positive post-chill carcasses. In addition, other studies did not find the decreasing in prevalence and number of *Campylobacter* after chilling (Reiter et al., 2005; Jozwiak et al., 2006). The difference in the change of prevalence might be because of the difference in the chilling system. One of the reasons might be the use of chlorination in chilling system. Generally, the result showed that 75% of post-chill chicken carcasses were *Campylobacter* positive which was comparable with those previously reported in the US and the UK which the prevalence ranged from 26 to 88% (Stern and Pretanik, 2006; Son et al., 2007; Simmons et al., 2008; Richardson et al., 2009; Hue et al., 2010; Powell et al., 2012; Schroeder, 2012). In Thailand, the prevalence of *Campylobacter* on post-chill carcasses ranged from 13.3-100% (Osiriphun et al., 2011; Chokboonmongkol et al., 2013).

When considering *Campylobacter* concentration on carcass after particular process from each slaughterhouse which had different evisceration and chilling process, the results showed that in slaughterhouse I and II which the evisceration was conducted manually, there was a 0.7 log increased and 0.2 log decreased concentration after evisceration, while in slaughterhouse III, where there was an automatic evisceration, approximately 0.5 log increasing was found. As for *Campylobacter* concentration on carcass after chilling, 1-2 log reduction of *Campylobacter* on post chilled carcasses was noted at slaughterhouse II and III which there was no use of chlorine in chilling process, while in slaughterhouse I which chlorine was added in chilling tank, there was 0.81 log reduction in concentration. A reduction of *Campylobacter* concentration in slaughterhouse I might be due to the use of chlorine in chilling water. Although no chemical substances were added in chilling tank, the level of *Campylobacter* decreased in slaughterhouses II and III. These findings suggested that aside from the use of chlorination in chilling tank, other factors such as an amount of chilling water per carcass etc. affecting the reduction of *Campylobacter* contaminated on post-chill carcasses.

Generally, the occurrence of *Campylobacter* from chicken carcass rinse depended on the *Campylobacter* status in the flock as detected in cecal content (Berrang et al., 2007), the results in our study showed that the occurrence in carcass rinse related to the occurrence in cecum. However, in flock F, which was the first flock slaughtered on that day, the occurrence of *Campylobacter* in carcass rinse was still high although the occurrence of *Campylobacter* in cecum was low. This might be due to the cross-contamination of *Campylobacter* that was remained on slaughtering line from the previous day. In addition to the occurrence of *Campylobacter* in cecum, the detection of *Campylobacter* in cloacal swab might be

used to determine *Campylobacter* status of the flock. The results in this present study showed that when the low occurrence of *Campylobacter* in cecum was observed, there was no detection of *Campylobacter* from clocal swab. Berrang et al. (2007) also revealed that the contamination of *Campylobacter* throughout the slaughtering process can be determined by the detection of *Campylobacter* in cloacal swab of live chickens as the arrival to the slaughterhouse.

For the concentration of *Campylobacter* in carcass rinses and ceca, our study as well as the study of Hue at al. (2011) and Rosenquist et al. (2006) found a positive correlation between the mean concentration of *Campylobacter* in ceca and *Campylobacter* concentration on carcasses. These findings suggested that the reduction of *Campylobacter* in ceca could reduce the amount of *Campylobacter* contaminating chicken carcasses. Even though the high concentration of *Campylobacter* in cecum and the high concentration in carcass were found in most flocks, the results in flock G showed low concentration of *Campylobacter* on carcass despite the high number of *Campylobacter* detected in cecum. There are some studies which found no association between *Campylobacter* in carcass rinse and in cecum or feces of chicken prior to slaughter (Allen et al., 2010 and Stern and Robach, 2003).

In this study, *flaA*-SVR types recovered from carcass rinses during slaughter were mostly related to the genotypes present in ceca or cloacal swabs. These findings were similar to previous studies (Chokboonmongkol et al., 2012; Norman et al, 2007) which found similar *Campylobacter* genotypes recovered from ceca and carcass rinses. Many studies showed that the number of *Campylobacter* subtypes decreased as chickens passed through the chilling process and only certain subtypes could be recovered from fully processed carcasses (Newell et al., 2001; Hiett et al.,

2002; Berrang et al., 2007; Hunter et al., 2009). Similarly, our study found a high number of *Campylobacter* subtypes in carcasses after defeathering but a number of subtypes decreased after chilling. Only 1-2 subtypes were found in post-chill carcasses except in flock B, which was contaminated with many subtypes. In flocks D, G and H, which were slaughtered as the first flock of the day were contaminated with a single subtype; therefore, slaughter order might affect the variation of subtypes contaminating carcasses during slaughtering process.

When contamination level of *Campylobacter* in three slaughterhouses was compared, slaughterhouse III, which only produced chicken products for export, had the lowest occurrence of *Campylobacter* (58%) and the mean concentration of *Campylobacter* on carcasses was also lower than other slaughterhouses (2.32 ± 0.49 log CFU/ml vs 2.98 ± 0.31 log CFU/ml and 2.66 ± 0.36 log CFU/ml). Moreover, this slaughterhouse was contaminated with only one subtype throughout the slaughtering process. The lower contamination rate might be contributed by more strict monitoring at critical control points during processing. Slaughterhouse II, which produced chicken products for both domestic consumption and export, had the highest occurrence of *Campylobacter* (84%) and the highest number of *Campylobacter* on carcasses (2.98 ± 0.31 log CFU/ml). This slaughterhouse was contaminated with 2-4 *Campylobacter* subtypes even the broiler flocks were slaughtered as the first flock of the day. Higher contamination level could be because of *Campylobacter* contaminated from previous day. Slaughterhouse I, which mainly produced products for consumption within the country, was contaminated with 1-10 *Campylobacter* subtypes. Different slaughtering procedures or monitoring system of critical control points might be contributed to numbers of subtypes found

during slaughtering process; therefore, a study on slaughtering factors influencing *Campylobacter* subtypes would be useful.

CONCLUSION AND SUGGESTION

The present study demonstrated that defeathering and evisceration steps were the critical process for *Campylobacter* contamination on broiler carcasses during slaughtering. Although inside-outside washing hardly reduced the contamination of *Campylobacter*, the level of contamination significantly decreased after chilling. Reduction of *Campylobacter* contamination on post-chill carcasses occurred in all slaughterhouses with or without the use of chlorine in chilling process. Therefore, it is important to determine effective control measures to reduce *Campylobacter* contamination in chickens especially at slaughter level.

Contamination rate of *Campylobacter* on carcass was likely involved with the occurrence of *Campylobacter* in cecum. Moreover, *flaA*-SVR types recovered from carcass rinse during slaughtering process were mostly related to *flaA*-SVR types in cecum and cloacal swab. It is worth noting that the status of *Campylobacter* in broiler flock affected the contamination of carcass at slaughterhouse. In *Campylobacter* positive flocks, 3-4 log CFU of *Campylobacter* per carcass can be found on chicken carcasses after chilling, thus increasing the chance of illness to consumers if cooking is not done properly. Since it is almost impossible to eliminate *Campylobacter* from carcasses of *Campylobacter* positive broiler flocks, first-line intervention strategies to prevent the introduction of *Campylobacter* into broiler farms are essential.

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APPENDIX

APPENDIX A

Culture media used for *Campylobacter* isolation

1. *Campylobacter* enrichment supplement (Exeter)

Antimicrobial agents	(mg/litre)
Amphotericin B	2
Cefoperazone	15
Polymyxin B	2500 IU
Rifampin	5
Trimethoprim	10

2. *Campylobacter* growth supplement

Typical formula	(mg/litre)
Sodium pyruvate	250
Sodium metabisulphite	250
Ferrous sulphate	250

* Complete Exeter broth includes nutrient broth No.2, lysed horse blood, *Campylobacter* growth supplement and *Campylobacter* selective supplement

3. *Campylobacter* blood-free selective agar base (mCCDA) (CM0739; Oxoid)

Typical Formula	(gm/litre)
Nutrient Broth No.2	25.0
Bacteriological charcoal	4.0
Casein hydrolysate	3.0
Sodium desoxycholate	1.0
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar	12.0

pH 7.4 ± 0.2 @25°C

4. CCDA selective supplement

Antimicrobial agents	(mg/litre)
Cefoperazone	32
Amphotericin B	10

Slaughter information of three slaughterhouses

Flock	Slaughterhouse	Slaughter order/ type of the operation	No. of birds killed in target house/ No. of flocks slaughtered in a day	Age of slaughter (days)/ average body weight (kg)	Occurrence of *Campylobacter* on carcass (%)	Mean concentration of *Campylobacter* on carcass (log CFU/ml)	No. of strains from which *flaA* was sequenced (No. of isolates)
A	I local	4/ manual evisceration	600/ 5-7	32 1.8	60	3.36	3 (31)
B	I local	3/ manual evisceration	600/ 5-7	32 1.8	80	2.09	10 (49)
C	I local	2/ manual evisceration	600/ 5-7	35 1.8	88	3.37	7 (34)
D	I local	1/ manual evisceration	600/ 5-7	32 1.8	84	2.13	1 (42)
E	II local and export	1/ manual evisceration	10,000/ 10-12	41 2.6	84	2.98	5 (37)
F	II local and export	1/ manual evisceration	10,000/ 10-12	40 2.5	84	-	6 (39)
G	III Export	1/ automated evisceration	10,000-30,000/ 10-15	42 2.7	100	2.32	1 (62)
H	III Export	1/ automated evisceration	10,000-30,000/ 10-15	40 2.5	16	-	1 (4)

VITA

Miss Suthida Muangnoicharoen was born on June 23, 1987 in Bangkok, Thailand. She got the degree in Doctor of Veterinary Medicine (D.V.M.) with first class-honor from the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2011. Shortly after, she continued her studies for a Master's degree at the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University in academic year 2011. Miss Suthida received the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej.