APPROPRIATE *CAMPYLOBACTER* DETECTION METHOD AND GENETIC VARIATION OF *CAMPYLOBACTER JEJUNI* IN BROILERS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Public Health
Department of Veterinary Public Health
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2018

วิธีการที่เหมาะสมในการตรวจแคมไพโลแบคเตอร์และความหลากหลายทางลักษณะพันธุกรรมของแคมไพโลแบคเตอร์ เจจูไนในไก่เนื้อ

น.ส.ณัฐณิชา เพชรศรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	APPROPRIATE *CAMPYLOBACTER* DETECTION METHOD AND GENETIC VARIATION OF *CAMPYLOBACTER JEJUNI* IN BROILERS
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Field of Study	Veterinary Public Health
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ณัฐณิชา เพชรศรี : วิธีการที่เหมาะสมในการตรวจแคมไพโลแบคเตอร์และความหลากหลายทางลักษณะพันธุกรรมของแคมไพโลแบคเตอร์ เจจูไนในไก่เนื้อ. (APPROPRIATE *CAMPYLOBACTER* DETECTION METHOD AND GENETIC VARIATION OF *CAMPYLOBACTER JEJUNI* IN BROILERS) อ.ที่ปรึกษาหลัก : ธราดล เหลืองทองคำ

แคมไพโลแบคเตอร์เป็นเชื้อสำคัญที่ทำให้เกิดโรคกระเพาะอาหารและลำไส้อักเสบในมนุษย์ซึ่งสาเหตุส่วนใหญ่มาจากการบริโภคเนื้อไก่ดิบหรือเนื้อไก่ที่ปรุงไม่สุก การศึกษาที่ผ่านมาพบว่าแคมไพโลแบคเตอร์ที่พบในโรงเชือดมีความใกล้เคียงกับแคมไพโลแบคเตอร์ที่แยกได้จากฟาร์มไก่เนื้อ ดังนั้นการตรวจสถานะการติดเชื้อแคมไพโลแบคเตอร์ของฝูงและการเรียงลำดับฝูงก่อนการเข้าเชือดจึงเป็นสิ่งสำคัญที่อาจช่วยป้องกันการปนเปื้อนเชื้อ แคมไพโลแบคเตอร์ระหว่างฝูงที่ให้ผลบวกและฝูงที่ให้ผลลบ อย่างไรก็ตามในปัจจุบันยังไม่มีการระบุวิธีการที่ชัดเจนและเหมาะสมสำหรับการตรวจเชื้อแคมไพโลแบคเตอร์ในระดับฟาร์ม เนื่องจากเชื้อแคมไพโลแบคเตอร์เป็นเชื้อที่เติบโตค่อนข้างยากและต้องการสภาวะที่จำเพาะในการเจริญเติบโต การตรวจที่ไม่เหมาะสมอาจนำไปสู่ผลลบลวงได้ ดังนั้นการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อ 1) ศึกษาชนิดตัวอย่างและวิธีการแยกเชื้อที่เหมาะสมสำหรับการตรวจเชื้อแคมไพโลแบคเตอร์ในฟาร์มไก่เนื้อ และ 2) ตรวจหาความสัมพันธ์ทางพันธุกรรมของเชื้อแคมไพโลแบคเตอร์ เจจูไน ระหว่างโรงเรือนไก่เนื้อภายในฟาร์มเดียวกัน โดยการศึกษานี้ประกอบด้วย 2 การทดลอง ได้แก่ การทดลองที่ 1 ทำการเก็บตัวอย่าง 3 ชนิดจากโรงเรือนเลี้ยงไก่เนื้อ 60 โรงเรือน ตัวอย่างดังกล่าวประกอบด้วย สวอปรองเท้า (boot swab) 60 ตัวอย่าง อุจจาระจากทวารร่วม (cloacal swab) 60 ตัวอย่าง และตัวอย่างอุจจาระสด (fresh fecal sample) 60 ตัวอย่าง จากนั้นทำการแยกเชื้อแคมไพโลแบคเตอร์จากตัวอย่างอุจจาระจากทวารร่วมและอุจจาระสดโดยวิธี Direct plating ลงบนอาหารเลี้ยงเชื้อชนิด modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA), modified Karmali (mKarmali), Preston และ Campy-cefex ส่วนตัวอย่างสวอปรองเท้านั้นทำการแยกเชื้อแคมไพโลแบคเตอร์ด้วยวิธี Direct plating ลงบนอาหารเลี้ยงเชื้อ 4 ชนิดตามที่กล่าวมาข้างต้นร่วมกับวิธี Selective enrichment โดยใช้อาหารเลี้ยงเชื้อที่ช่วยส่งเสริมการเจริญเติบโตชนิด Bolton, Preston, Exeter และ blood-free Bolton การวิเคราะห์ความแตกต่างของอัตราการแยกเชื้อแคมไพโลแบคเตอร์จะใช้สถิติชนิด McNemar test *($p<0.05$)* ในการวิเคราะห์ชนิดตัวอย่างที่เหมาะสมสำหรับการตรวจเชื้อแคมไพโลแบคเตอร์จะทำการเปรียบเทียบอัตราการแยกเชื้อแคมไพโลแบคเตอร์จากวิธี Direct plating เท่านั้น ผลการทดลองพบว่าตัวอย่างสวอปรองเท้าให้อัตราการแยกเชื้อสูงที่สุดที่ 26.7% รองลงมาคือตัวอย่างอุจจาระจากทวารร่วม (20%) และตัวอย่างอุจจาระสด (15%) จากการเปรียบเทียบความสามารถในการแยกเชื้อแคมไพโลแบคเตอร์โดยอาหารเลี้ยงเชื้อแต่ละชนิด พบว่า mCCDA ให้ผลการแยกเชื้อแคมไพโลแบคเตอร์จากตัวอย่างฟาร์มไก่เนื้อได้ดีกว่าอาหารเลี้ยงเชื้อชนิดอื่นๆ นอกจากนี้ยังพบว่าการใช้อาหารเลี้ยงเชื้อชนิด Preston ควบคู่ไปกับอาหารเลี้ยงเชื้อชนิด mCCDA สามารถเพิ่มอัตราการแยกเชื้อแคมไพโลแบคเตอร์ได้ดียิ่งขึ้น สำหรับการทดลองที่ 2 นั้นทำการเก็บตัวอย่างและเพาะแยกเชื้อแคมไพโลแบคเตอร์จากตัวอย่างสวอปรองเท้าจากฟาร์มไก่เนื้อจำนวน 12 ฟาร์ม รวมทั้งสิ้น 60 โรงเรือน (5 โรงเรือนต่อฟาร์ม) จากนั้นทำการศึกษาลักษณะทางพันธุกรรมของเชื้อแคมไพโลแบคเตอร์ เจจูไน ที่แยกได้จากโรงเรือนไก่เนื้อภายในฟาร์มเดียวกันด้วยวิธี Comparative Genomic Fingerprint 40 (CGF40) ผลการทดลองพบว่าภายในฟาร์มไก่เนื้อมีทั้งโรงเรือนที่ให้ผลบวกและผลลบต่อเชื้อแคมไพโลแบคเตอร์ เจจูไน ถึงแม้ว่าลักษณะพันธุกรรมของเชื้อแคมไพโลแบคเตอร์ เจจูไน ที่แยกได้จากฟาร์มไก่เนื้อแต่ละฟาร์มจะมีลักษณะเฉพาะที่แตกต่างกันไป แต่ลักษณะพันธุกรรมของเชื้อแคมไพโลแบคเตอร์ เจจูไน ที่แยกได้จากโรงเรือนที่อยู่ในฟาร์มเดียวกันจะค่อนข้างมีความใกล้เคียงกัน จากผลการศึกษาแสดงให้เห็นว่าการตรวจสถานะการติดเชื้อแคมไพโลแบคเตอร์ในฟาร์มไก่เนื้อควรเก็บตัวอย่างสวอปรองเท้าจากโรงเรือนมากกว่า 1 โรงเรือนภายในฟาร์มเดียวกันและทำการเพาะแยกเชื้อแคมไพโลแบคเตอร์จากตัวอย่างสวอปรองเท้าด้วยวิธี Direct plating ลงบนอาหารเลี้ยงเชื้อชนิด mCCDA ควบคู่ไปกับอาหารเลี้ยงเชื้อชนิด Preston ซึ่งการตรวจเชื้อแคมไพโลแบคเตอร์ในระดับฟาร์มด้วยวิธีที่เหมาะสมและจัดลำดับฝูงก่อนการเข้าเชือดจะช่วยลดการปนเปื้อนของเชื้อแคมไพโลแบคเตอร์ในระหว่างกระบวนการผลิตได้

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
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5875307231 : MAJOR VETERINARY PUBLIC HEALTH

KEYWORD: Campylobacter Campylobacter jejuni Broiler farm Sample type Isolation method Genetic

Natnicha Phetsri : APPROPRIATE *CAMPYLOBACTER* DETECTION METHOD AND GENETIC VARIATION OF *CAMPYLOBACTER JEJUNI* IN BROILERS. Advisor: TARADON LUANGTONGKUM, D.V.M., Ph.D.

Campylobacter has been known as an important cause of gastroenteritis in humans. The primary source is raw or undercooked poultry meat. Previous study showed that *Campylobacter* isolated from slaughterhouses were associated with *Campylobacter* isolated from broiler farms. Thus, detection of *Campylobacter* flock status and sorting flocks prior to slaughtering processes may help prevent *Campylobacter* contamination between *Campylobacter*-positive and negative flocks during slaughtering processes. Unfortunately, appropriate sample type and isolation method have not been clearly specified for *Campylobacter* detection at the farm level. Because *Campylobacter* are fastidious bacteria, improper detection method can attribute to false negative results. Therefore, the objectives of this study were 1) to identify appropriate sample type and isolation method for *Campylobacter* detection in broilers and 2) to determine genetic relatedness of *C. jejuni* from different broiler houses in the same farm. This study consists of 2 experiments. In the first experiment, three types of sample were obtained from 60 broiler houses including 60 boot swabs, 60 cloacal swabs and 60 fresh fecal samples. *Campylobacter* were isolated from cloacal swab and fresh fecal samples by direct plating method using modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA), modified Karmali (mKarmali), Preston agar and Campy-cefex agar. Isolation of *Campylobacter* from boot swabs was conducted by both direct plating method and selective enrichment method using Bolton broth, Preston broth, Exeter broth and blood-free Bolton broth as selective enrichment. To determine the most suitable sample type, only *Campylobacter* isolation rates from direct plating method were compared. The difference in *Campylobacter* isolation rate was analyzed by McNemar test *($p<0.05$)*. The results showed that boot swabs provided the highest *Campylobacter* isolation rate at 26.7%, followed by cloacal swabs (20%) and fresh feces (15%). Among the media used, mCCDA is better than the other media tested for *Campylobacter* isolation from farm samples. In addition, our results revealed that using Preston agar as the second media along with mCCDA could significantly increase *Campylobacter* isolation rate. For the second experiment, boot swabs were obtained from 60 houses of 12 broiler farms (5 houses/farm). Genetic relatedness of *C. jejuni* isolated from each broiler house within each broiler farm was examined by Comparative Genomic Fingerprint 40 (CGF40). The results showed that *Campylobacter*-positive and *Campylobacter*-negative houses were observed in each broiler farm. Although specific CGF40 patterns were noticed among *C. jejuni* isolated from each broiler farm, *C. jejuni* from broiler houses in the same farm had quite similar CGF40 patterns. According to our findings, we suggest that boot swabs should be collected from a few or more houses in the same farm and *Campylobacter* detection should be conducted by direct plating of boot swab samples onto both mCCDA and Preston agar. *Campylobacter* detection at the farm level by using appropriate sample type and isolation method is important to correctly obtain *Campylobacter* flock status and sort flocks prior to slaughtering, which can significantly help decrease *Campylobacter* cross-contamination during slaughtering processes.

Field of Study:	Veterinary Public Health	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to thank all those who involved with this dissertation. Firstly, I would like to express my sincere gratitude to Dr. Taradon Luangtongkum, my thesis advisor, for his patience, being supportive, provided me with professional guidance and suggestions through the course of this research. My sincere thanks also goes to Dr. Nipa Chokesajjawatee and staffs at National Center for Genetic Engineering and Biotechnology who gave access to the laboratory, research facilities and thanks for thier suggestions for CGF40 technique. I would also like to thank all committee members for their valuable comments and suggestions. Besides, I would like to thank my colleagues at Campylobacter Research Laboratory and my friend at Department of Veterinary Public Health for their help, suggestions and friendship. Most importantly, I would like to thank my parents, for their continuous support, understanding, and love that they have given me all the time. Finally, this dissertation would not have been possible without the financial support of National Bureau of Agricultural Commodity and Food Standards.

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LIST OF ABBREVIATIONS

23S rRNA	23S Ribosomal Ribonucleic acid
Bp	Base pair
°C	degree(s) Celsius
C. coli	*Campylobacter coli*
C. jejuni	*Campylobacter jejuni*
dNTP	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
FSIS	Food Safety and Inspection Service
ISO	International Organization for Standardization
mCCDA	Modified Charcoal-Cefoperazone-Deoxycholate
mKarmali	Modified Karmali
min	minute(s)
NCTC	National Collection of Type Cultures
OIE	Office International des Epizooties

PCR	Polymerase chain reaction
spp.	species
Taq	Thermus aquaticus
USDA	U.S. Department of Agriculture

CHAPTER I
INTRODUCTION

Campylobacter has been recognized as one of the most important foodborne pathogens. *Campylobacter* infection in humans known as campylobacteriosis is widely associated with poultry meat and poultry products consumption (Lindmark et al., 2009; van Gerwe, 2012; Skarp et al., 2016). In addition, foodborne campylobacteriosis causes economic loss around £50 million in the United Kingdom during 2008-2009 (Tam and O'Brien, 2016). European food safety authority (EFSA) reported that most gastroenteritis cases in humans in Europe were caused by *Campylobacter.* Approximately 200,000 foodborne campylobacteriosis cases were reported in 2013 and the number of cases seems to continuously increase (Eurosurveillance editorial, 2015). Most campylobacteriosis cases are caused by *Campylobacter jejuni* and *Campylobacter coli* (Coker et al., 2002; Lindmark et al., 2009; Kaakoush et al., 2015; Skarp et al., 2016). In Thailand, *Campylobacter* is the most common cause of acute bacterial diarrhea and the leading cause of mortality among children under 5-year-old (Padungtod and Kaneene, 2005; Bodhidatta et al., 2010; Samosornsuk et al., 2015).

Campylobacter-contaminated chicken meat is the main source of human infection (Skarp et al., 2016). The previous study found that *Campylobacter* isolates from broiler farms were closely related to *Campylobacter* isolated from chicken

meats (Prachantasena et al., 2016). To prevent cross-contamination at processing steps, *Campylobacter* detection should be conducted at broiler flocks prior to slaughtering. *Campylobacter* detection protocols for poultry products and food are now available, while an official protocol for *Campylobacter* monitoring at broiler farm level is not available. It is important to detect and prevent *Campylobacter* at the farm level because it can help reduce *Campylobacter* contamination on chicken meats and products (Franz et al., 2012; Georgiev et al., 2017).

Detection of *Campylobacter* flock status is essential for sorting flocks before slaughtering process. Several studies suggested that knowing flock status and sorting prior to slaughtering could prevent or reduce *Campylobacter* contamination (Bronzwaer et al., 2009; Berghaus et al., 2013; Haas et al., 2017). Because *Campylobacter* are fastidious bacteria, improper detection method can lead to false negative results. The suitable sample type and isolation method are important for detection of *Campylobacter* status of broiler farms. *Campylobacter*-negative flocks should be transported into slaughtering process before *Campylobacter*-positive flocks in order to prevent cross-contamination between positive and negative flocks (Wagenaar et al., 2006; Havelaar et al., 2007; Silva et al., 2011).

In terms of sample types, a variety of samples including cloacal swab, fresh feces, sock/boot swab, etc. were used for *Campylobacter* detection at broiler farms (Patriarchi et al., 2009; Thakur et al., 2013; Vidal et al., 2013; Ingresa-Capaccioni et al.,

2015a; Seliwiorstow et al., 2015). For *Campylobacter* isolation method, selective enrichment method such as ISO 10272-1 protocol was commonly used (Patriarchi et al., 2009; Habib et al., 2011; Ingresa-Capaccioni et al., 2015a). The ISO 10272-1 protocol seemed to be suitable for detection of *Campylobacter* from low background microorganism samples. Several studies found that the ISO 10272-1 method was not suitable for all sample types especially samples with high background microorganisms (Habib et al., 2011; Ugarte-Ruiz et al., 2012; Seliwiorstow et al., 2016). Since each isolation method uses different types of agar and enrichment broth, which can affect *Campylobacter* isolation rates (Potturi-Venkata et al., 2007; Kiess et al., 2010; Vaz et al., 2014), it is necessary to select a proper detection method to obtain reliable information on *Campylobacter* flock status.

In addition to sample type and isolation method, sampling unit for sample collection at the farm level is also important. Collection of samples from only one house might not actually represent *Campylobacter* status of the farm because some houses in a *Campylobacter*-negative farm might be positive for *Campylobacter* and the variance of *Campylobacter jejuni* was also reported among broiler houses (Kapperud et al., 1993; Evans and Sayers, 2000; Thakur et al., 2013; Vidal et al., 2016). The genetic diversity of *C. jejuni* isolates among broiler farms should be considered when establishing monitoring and controlling program to understand *Campylobacter* populations in broiler farms. However, the study about epidemiological pattern of

Campylobacter jejuni among broiler houses in the same broiler farm in Thailand is limited.

Hence, proper sample type, isolation method and sampling unit are required to obtain accurate results of *Campylobacter* status of the farm. Although several detection strategies are recommended, the strategies specific for *Campylobacte*r detection at a farm level in Thailand are not available. Therefore, this study was conducted to identify appropriate sample type and isolation method for *Campylobacter* detection at a farm level and to determine genetic relatedness of *C. jejuni* isolated from different broiler houses in the same broiler farms.

CHAPTER II
LITERATURE REVIEW

2.1 General characterization of *Campylobacter*

Campylobacter is gram negative bacteria with curve or spiral shape belonging to *Campylobacteriaceae* family. *Campylobacter* are fastidious bacteria. They require specific conditions to grow. They are sensitive to high oxygen. The optimal growth condition is 41.5°C under 5% O_2, 10% CO_2 and 85% N_2, which is known as microaerobic condition (ISO, 2006). They cannot grow at 25°C under microaerobic conditions and at 42°C under aerobic conditions (ISO, 2006). *Campylobacter* species are oxidase and catalase positive. Although *Campylobacter* colony is flat, round and smooth edge, they are different in color depending on culture media. *Campylobacter* colony on charcoal-based agar is grayish with metallic sheen, while *Campylobacter* colony on blood-based agar is slightly pink(ISO, 2006). Among *Campylobacter* species, *C. jejuni* and *C. coli* are the most common causes of illness in humans (Lindmark et al., 2009; van Gerwe, 2012; Skarp et al., 2016).

2.2 Campylobacteriosis

Campylobacteriosis is the gastrointestinal disease in humans caused by *Campylobacter* spp., especially *C. jejuni* and *C. coli* (Padungtod and Kaneene, 2005; Samosornsuk et al., 2015; Skarp et al., 2016). Humans are usually infected with *Campylobacter* via consumption of undercooked chicken meat, poultry products

and contaminated water (Kaakoush et al., 2015). *Campylobacter* cause illnesses such as fever, watery or bloody diarrhea and abdominal cramps in humans. These symptoms are usually recovered within a few days. In some cases, complications such as reactive arthritis and Guillain-Barré Syndrome may occur after several weeks of infection (Allos; Nachamkin et al., 1998; Huizinga et al., 2015).

2.3 *Campylobacter* in broiler flocks

Campylobacter prevalence in Thai broiler flocks was 57% with 10-100% of within flock prevalence (Prachantasena et al., 2017). In European countries, *Campylobacter* prevalence at broiler farm could be high as 80% (Lawes et al., 2012; Allain et al., 2014; Torralbo et al., 2014). Compared to other countries, *Campylobacter* prevalence at the farm level in Japan and Canada was slightly lower, which accounted for 47% and 35%, respectively (Arsenault et al., 2007; Haruna et al., 2012). Among *Campylobacter* isolated from broiler farms, *C. jejuni* is the most common species followed by *C. coli* (Meeyam et al., 2004; Lawes et al., 2012; Allain et al., 2014; Torralbo et al., 2014; Prachantasena et al., 2017).

Generally, broiler flocks were *Campylobacter*-negative until 2-3 weeks of age. After that, flocks may become *Campylobacter*-positive during the rearing period until slaughtering (Bull et al., 2006; Ingresa-Capaccioni et al., 2015a). The highest detection rate was usually found at the end of the rearing period (Ingresa-Capaccioni et al., 2015b). *Campylobacter* isolated from broiler farms were found to be related to

Campylobacter isolated from poultry meat at slaughterhouses (Powell et al., 2012; Prachantasena et al., 2016). Interestingly, *Campylobacter* could be detected at slaughtering processes even though those flocks were negative to *Campylobacter* at broiler farm level (Wieczorek and Osek, 2015). Since cross-contamination of *Campylobacter* might occur during processing steps, detection of broiler flock status and sorting flocks prior to slaughtering could prevent and/or reduce *Campylobacter* cross-contamination on poultry meat at processing steps (Bronzwaer et al., 2009; Berghaus et al., 2013; Haas et al., 2017).

2.4 *Campylobacter* detection method

Campylobacter in broiler farms can be detected from cloacal swab, fresh feces, boot swab, dust swab and even water (Bull et al., 2006). However, there are some studies evaluated the suitable sample type for *Campylobacter* surveillance at broiler farms (Ingresa-Capaccioni et al., 2015a; Seliwiorstow et al., 2015; Vidal et al., 2016). In general, sample types that are widely used for *Campylobacter* detection at broiler farm level are cloacal swabs, fresh feces and boot swabs (Hansson et al., 2004; Patriarchi et al., 2009; Thakur et al., 2013; Vidal et al., 2013; Ingresa-Capaccioni et al., 2015a).

Direct plating method and selective enrichment method can be used for *Campylobacter* isolation from broiler farm samples. Although selective enrichment method such as ISO 10272-1 protocol (ISO, 2006) is generally used for

Campylobacter isolation, it might promote the growth of other background microorganisms, which may obscure *Campylobacter* colonies. For high *Campylobacter*-contaminated samples such as cloacal swabs and fresh feces, direct plating method may be the most suitable isolation method (Kiess et al., 2010).

Several types of agar are recommended and commercially available for direct plating method such as modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA), mKarmali agar, Preston agar and Campy-cefex agar. mCCDA and mKarmali agar are charcoal-based selective agar, while Preston agar and Campy-cefex agar are blood-based selective agar (ISO, 2006). Each type of agar has different supplements and antimicrobial agents used for inhibiting background microorganisms. mCCDA is a commonly used medium due to its selectivity. Also, *Campylobacter* colony on the media is easy to observe (Rodgers et al., 2012; Gharst et al., 2013). Nonetheless, it might not be suitable for all types of samples. Preston agar was found to be more sensitive for *Campylobacter* isolation from feces and litter samples than mCCDA, while mCCDA is more efficient than Preston agar for *Campylobacter* isolation from cloacal swab sample (Vaz et al., 2014). In addition, Campy-cefex agar gave higher *Campylobacter* isolation rates than mCCDA for carcass rinse samples and fresh fecal samples (Oyarzabal et al., 2005; Potturi-Venkata et al., 2007; Thakur et al., 2013).

Similar to selective agar media, selective enrichment broth such as Bolton broth, Preston broth, Exeter broth, etc. also have different supplements and

antimicrobial agents for inhibition of non-*Campylobacter* background microorganisms. Generally, most types of broth have blood as an important ingredient. Blood will quench toxic oxygen in the air, which is toxic to *Campylobacter*. However, blood may promote the growth of non-*Campylobacter* microorganisms. So, *Campylobacter* isolation protocol of the United States Department of Agriculture for Food Safety and Inspection Service (USDA-FSIS) recommends that enrichment broth without blood should be used (Gharst et al., 2013; USDA, 2013). Selective enrichment method is mainly used for *Campylobacter* isolation from low *Campylobacter*-contaminated samples. Bolton broth has been recommended by 10272-1 protocol for *Campylobacter* isolation from food and raw products (ISO, 2006). Although Bolton broth might be suitable for *Campylobacter* isolation from low background samples such as food and cooked meat, it is not suitable for high background samples. It was suggested in the previous study that Preston broth provided higher isolation rates than Bolton broth for *Campylobacter* isolation from high background samples such as raw meat, raw chicken products and raw milk (Gharst et al., 2013).

2.5 Genetic characterization of *Campylobacter jejuni*

Presently, several molecular genotyping methods such as amplified fragment length polymorphism (AFLP), flaA typing, flaA restriction fragment length polymorphism (flaA-RFLP), repetitive element sequence-based PCR (rep-PCR),

multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) has been widely used for *C. jejuni* characterization (Lindstedt et al., 2000; Wassenaar and Newell, 2000; Wagenaar et al., 2001; Schouls et al., 2003; Wardak and Jagielski, 2009; Behringer et al., 2011; Pendleton et al., 2013). Among these available genotyping methods, PFGE was found to have higher discriminatory power than other methods and it is also recognized as gold standard for *Campylobacter* characterization (Colles et al., 2003; Wardak and Jagielski, 2009). Although MLST is widely used for *Campylobacter* genetic characterization and source tracking (Noormohamed and Fakhr, 2014), this method is expensive for routine and large scale surveillance (Djordjevic et al., 2007). Therefore, comparative genomic fingerprint 40 (CGF40) was evaluated to be an alternative method for genetic characterization of *C. jejuni* isolates (Taboada et al., 2012). CGF40 was found to have a high discriminatory power and the results from this method also had a good concordance with MLST (Taboada et al., 2008; Clark et al., 2012; Taboada et al., 2013).

2.6 Comparative Genomic Fingerprint 40 (CGF40)

CGF40 is genotyping method developed for *C. jejuni* genetic characterization using multiplex PCR technique. Genetic patterns by CGF40 is based on the presence of 40 selected genes, which were selected from three microarray-based comparative genome sets. These genes were selected from unbiased genes (very high presence or absence rates genes were excluded), genes distributed throughout genome, including

accessory genes from major hypervariable regions. In addition, these genes were present in two or more tested *C. jejuni* whole genome sets. Besides, they also could indicate relationship among strains (Taboada et al., 2004; Taboada et al., 2012).

CHAPTER III
MATERIALS AND METHODS

This study consists of 2 experiments. The first experiment is to identify appropriate sample type and isolation method for *Campylobacter* detection at broiler farms. This experiment is comprised of 3 phases including sample collection (Phase I), *Campylobacter* isolation (Phase II) and *Campylobacter* confirmation and identification (Phase III). For the second experiment, genetic relatedness of *C. jejuni* in different broiler houses was determined. This experiment is composed of 4 phases including sample collection (Phase I), *Campylobacter* isolation (Phase II), *Campylobacter* confirmation and identification (Phase III) and genetic characterization of *C. jejuni* isolates (Phase IV). The schematic outline of the workflow of this study is shown in Figure 1.

Several types of media are recommended and commercially available for *Campylobacter* isolation such as modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA), Karmali agar, Preston agar and Campy-cefex agar. Although mCCDA is commonly used agar for detection of *Campylobacter*, a few studies found that mCCDA had less sensitivity than Preston agar and Campy-cefex agar for isolation of *Campylobacter* from broiler farm samples and carcass rinse samples, respectively (Oyarzabal et al., 2005; Vaz et al., 2014). Therefore, mCCDA was compared with others three commercial selective media for *Campylobacter* isolation in this study.

Although Bolton broth and blood-free Bolton broth have been recommended by ISO and USDA standard method (ISO, 2006; USDA, 2013), respectively, Preston broth and Exeter broth has been found to be suitable for *Campylobacter* detection from high background samples such as samples from farms, raw products, etc. (Gharst et al., 2013; Vidal et al., 2013; Seliwiorstow et al., 2014). Therefore, these 4 types of selective enrichment broths were compared in this study.

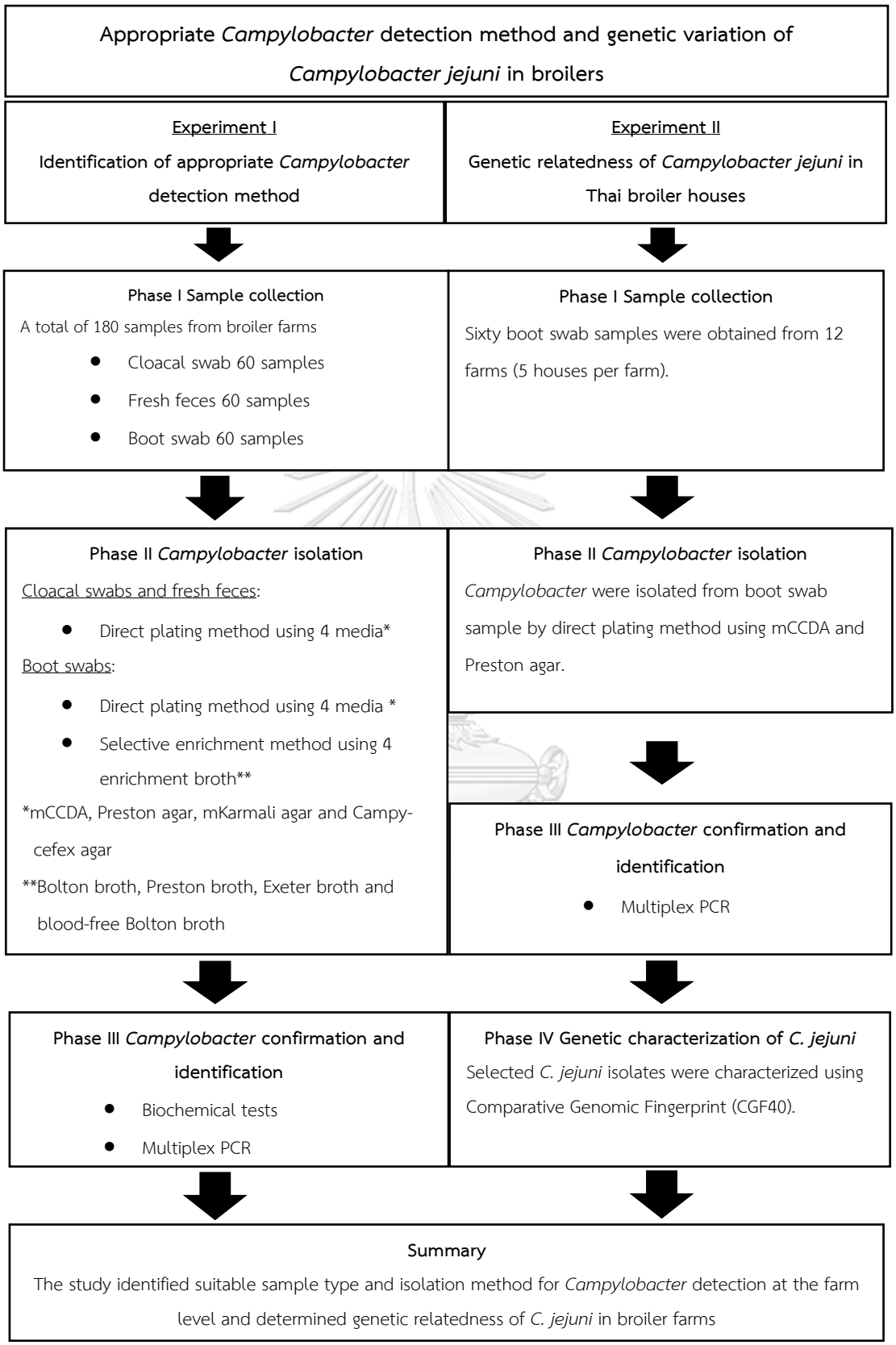


Figure 1 Schematic outline of the workflow of this study

3.1 Appropriate sample type and isolation method for *Campylobacter* detection from broiler farms

The first experiment consists of 3 phases including sample collection (phase I), *Campylobacter* isolation (phase II) and *Campylobacter* confirmation and identification (phase III). Three different types of samples including cloacal swabs, fresh fecal samples and boot swab samples were obtained from 60 broiler houses with previous history of *Campylobacter*-positive flocks. All visited farms were located in the central region of Thailand and not more than 3 hours away from the laboratory. *Campylobacter* from each sample type was isolated using different isolation methods. Suspected *Campylobacter* colonies were confirmed and identified by biochemical tests and multiplex PCR.

3.1.1 Sample collection

Broiler farms with previous history of *Campylobacter*-positive flocks were included in the first experiment. Since high *Campylobacter* detection rate was found when chickens were close to market age (around 6 weeks), samples were obtained from broiler chickens with 4-5 weeks of flock age (one week before the birds were transferred to slaughterhouse)s as recommended by the previous studies (Potturi-Venkata et al., 2007; Ugarte-Ruiz et al., 2013; Vidal et al., 2013; Seliwiorstow et al., 2014; Ingresa-Capaccioni et al., 2015a; Seliwiorstow et al., 2015). A total of 180 samples including 60 cloacal swabs (600 chickens), 60 fresh fecal samples (600 piles

of fecal droppings) and 60 pairs of boot swab were obtained from 60 broiler houses. All samples were placed on ice during transportation and analyzed within 2-3 hours after collection.

3.1.2 *Campylobacter* isolation

To compare *Campylobacter* isolation rate of each sample type and isolation media used for *Campylobacter* detection from broiler farm samples, *Campylobacter* were isolated from cloacal swab and fresh fecal samples by direct plating method using four types of media including mCCDA, mKarmali agar, Preston agar and Campy-cefex agar (Table 1), whereas *Campylobacter* were isolated from boot swab samples by both direct plating method using four types of media and selective enrichment method using four types of selective enrichment broths (Table 2).

3.1.2.1 Cloacal swab samples

Ten cloacal swabs were obtained from ten broiler chickens in each broiler house. Cloacal swab samples were obtained by insertion of sterile cotton swabs into chickens cloaca. After collection, each cotton swab was put into a sterile tube that contained sterile saline (0.85% NaCl). Ten cloacal swabs from each house were pooled to form a single sample. Pooled samples were homogenized and loopful of pooled samples was streaked onto four types of media including mCCDA, mKarmali agar, Preston agar and Campy-cefex agar (Table 1). All inoculated plates were placed into anaerobic jar and incubated at 42°C for 48±2 hours under microaerobic

conditions (5% O_2, 10% CO_2, 85% N_2) using Anaero - MicroAeroPack System® sachets (Mitsubishi Gas Chemical Co. Inc, Japan). After incubation, morphology of *Campylobacter* colonies was observed. *Campylobacter* colonies on blood-based and charcoal-based are shown in Appendix C.

3.1.2.2 Fresh fecal samples

Ten piles of fresh fecal droppings were collected from each broiler house and pooled into one sample in a sterile plastic pot containing sterile saline (0.85% NaCl) at broiler farms. *Campylobacter* were isolated from pooled fresh fecal samples by the method and conditions similar to those used for cloacal swab samples (3.1.2.1).

3.1.2.3 Boot swab samples

Boot swab samples were obtained by wearing shoe cover and walking through broiler houses. Pairs of shoe cover were put into plastic bags that contained sterile saline (0.85% NaCl). *Campylobacter* were isolated from boot swab samples by direct plating method using four types of media including mCCDA, mKarmali, Preston gar and Campy-cefex. Four loofuls of saline were streaked onto mCCDA, mKarmali, Preston gar and Campy-cefex, then incubated as described earlier.

In addition to direct plating method, *Campylobacter* were isolated from boot swab samples by selective enrichment method using Bolton broth, Preston broth, Exeter broth and blood-free Bolton broth. For Bolton broth, Preston broth and Exeter broth, samples were incubated at 37°C for 4-6 hours and then at 42°C for 44 hours

under microaerobic conditions using Anaero - MicroAeroPack System® sachets (Mitsubishi Gas Chemical Co. Inc, Japan) (ISO, 2006). Additionally, *Campylobacter* were isolated from boot swab samples by USDA protocol, which recommends blood-free Bolton broth as selective enrichment broth (USDA, 2013). For blood-free Bolton broth, samples were incubated at 42°C for 48±2 hours under microaerobic conditions using Anaero - MicroAeroPack System® sachets (Mitsubishi Gas Chemical Co. Inc, Japan) (USDA, 2013). After enrichment step, loopfuls of all enrichment broths were streaked onto mCCDA, mKarmali, Preston agar and Campy-cefex agar. The inoculated plates were incubated under microaerobic conditions. After incubation, morphology of *Campylobacter* colonies was observed. A summary of *Campylobacter* isolation methods is shown in Figure 2.

Table 1 *Campylobacter* isolation media

Supplement	Selective media (concentration mg/liter)			
	mCCDA	mKarmali	Preston	Campy-cefex
Amphotericin B	32	32	-	-
Cefoperazone	10	10	-	33
Cycloheximide	-	-	100	200
Polymixin B	-	-	5,000 IU	-
Rifampicin	-	-	10	-
Trimethoprim	-	-	10	-
Vancomycin	-	20	-	-
Sodium pyruvate	250	100	-	500
Peptone	-	-	10,000	10,000
Lab-Lemco peptone	-	-	10,000	5,000
Glucose	-	-	-	10,000
Sodium chloride	-	-	5,000	5,000
Nutrient Broth No.2	25,000	-	-	-
Casein hydrolysate	3,000	-	-	-
Sodium desoxycholate	1,000	-	-	-
Sodium bisulphate	-	-	-	200
Ferrous sulphate	250	-	-	500
Brucella base	-	-	-	43,000
Columbia blood base	-	39,000	-	-
Agar base	12,000	-	-	15,000
Sheep blood	-	-	50 (ml)	50 (ml)
Activated charcoal	-	4,000	-	-
Bacteriological charcoal	4,000	-	-	-

Table 2 *Campylobacter* selective enrichment broth

Supplement	Selective enrichment broth (concentration mg/liter)			
	Bolton broth	Preston broth	Exeter broth	Blood-free Bolton broth
Amphotericin B	-	-	2	-
Cefoperazone	20	-	15	40
Cycloheximide	50	100	-	100
Polymixin B	-	5,000	2500 (IU)	-
Rifampicin	-	10	5	-
Trimethoprim	20	10	10	40
Vancomycin	20	-	-	40
Lab-Lemco Power	-	10,000	10,000	-
Peptone	-	10,000	10,000	-
Meat peptone	10,000	-	-	10,000
Lactalbumin hydrolysate	5,000	-	-	5,000
Yeast extract	5,000	-	-	5,000
Sodium chloride	5,000	-	-	5,000
Alpha-ketoglutaric acid	1,000	-	-	1,000
Sodium pyruvate	500	250	250	500
Sodium metabisulphite	500	250	250	500
Sodium carbonate	600	-	-	600
Ferrous sulphate	-	250	250	
Sodium chloride	-	5,000	5,000	-
Haemin	10	-	-	10
Sheep blood	50 (ml)	50 (ml)	50 (ml)	-

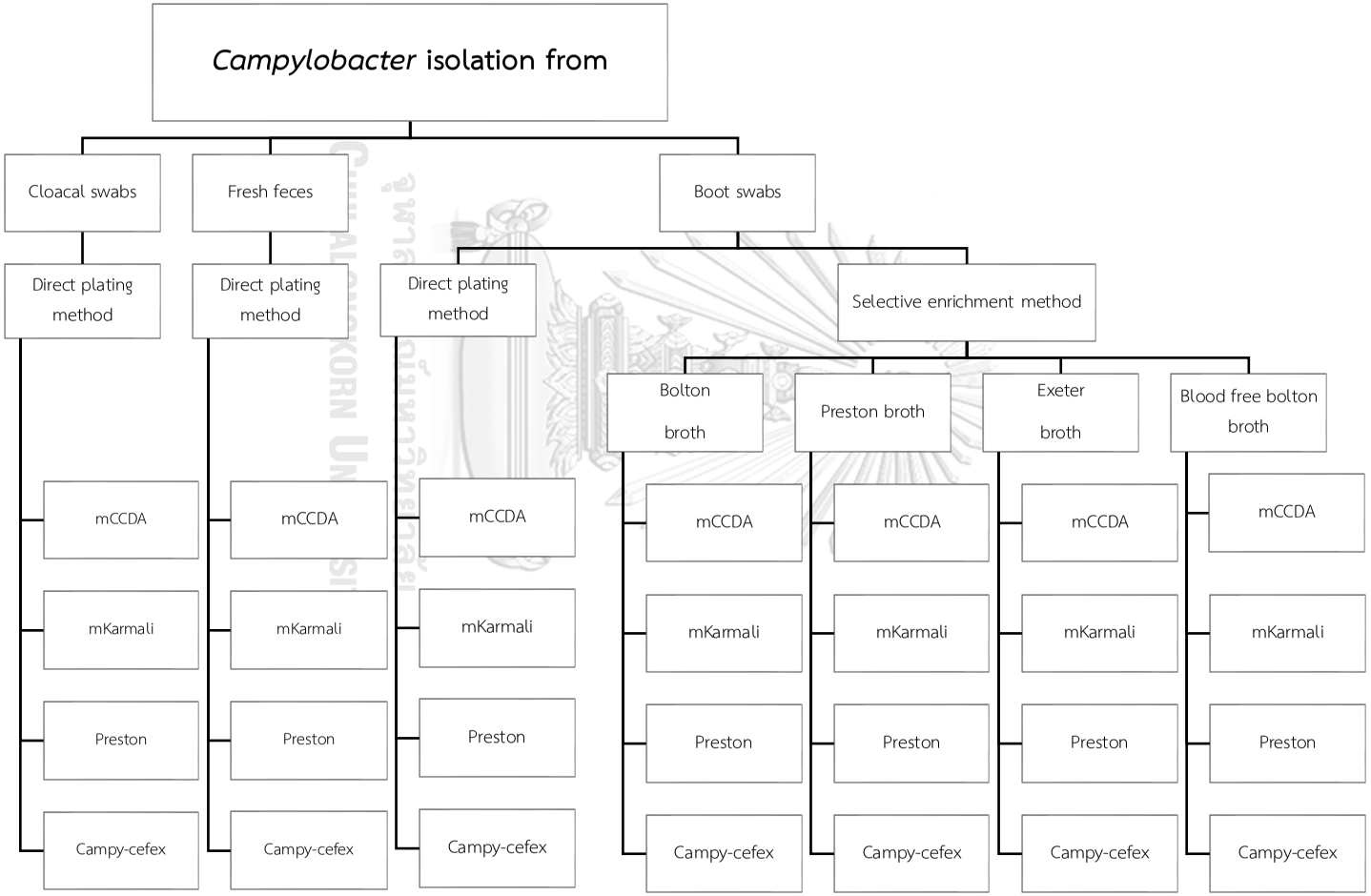


Figure 2 A summary of *Campylobacter* isolation methods that were performed in this study

3.1.3 *Campylobacter* confirmation and identification

Approximately 2-4 suspected colonies were streaked onto non-selective blood agar (Columbia blood agar, CM 331, Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and incubated at 42°C for 48±2 hours under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) using Anaero - MicroAeroPack System® sachets (Mitsubishi Gas Chemical Co. Inc, Japan). Suspected colonies were confirmed by biochemical tests including catalase test, oxidase test and hippurate hydrolysis test (Appendix D). In addition, presumptive colonies were identified to *Campylobacter* species level by multiplex PCR according to the previously published protocol (Wang et al., 2002) with some modifications (Table 3).

3.1.3.1 Multiplex PCR

a. DNA extraction

For DNA extraction, a small amount of *Campylobacter* colony was put into microcentrifuge tubes containing 100 µl of sterilized distilled water and then homogenized using vortex. The microcentrifuge tubes were heated in boiling water for 10 min, then they were immediately placed on ice and centrifuged at 18,928 ×g (13,000 rpm) for 5 min. After centrifugation, the supernatant was transferred to a new microfuge tube and used as DNA template. DNA templates were kept frozen at -20°C.

b. DNA amplification

Campylobacter colonies were confirmed and identified to genus and species level by multiplex PCR technique using 3 pairs of primers (Table 3) according to Wang et al. (2002) with some modifications. DNA amplification was performed in a total volume of 25 µl containing 12.5 µl of KAPA Taq ReadyMix (Kappa Biosystems, Wilmington, Massachusetts, USA); 1.25 µl of each 0.5 µM *C. jejuni* forward and reverse primers; 2.5 µl of each 1 µM *C. coli* forward and reverse primers and 0.5 µl of each 0.2 µM 23S rRNA forward and reverse primers and 2.5 µl DNA template. The volume of each reaction was adjusted by adding 1.5 µl of deionized water/nuclease-free water to make 25 µl. DNA amplification was conducted in a thermocycler with initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification; denaturation at 95°C for 0.5 min, annealing at 57°C for 0.5 min, extension at 72°C for 0.5 min and final extension step at 72°C for 7 min. PCR-amplified products were analyzed in 1.5% agarose gel electrophoresis.

c. Gel electrophoresis

Three µl of PCR-amplified products were loaded into 1.5% agarose gel and placed into electrophoresis chamber. Electrophoresis was carried out at 90 volts for 45 min using 1× TAE as a buffer. The size of PCR products is shown in Table 3.

Table 3 Primers for *Campylobacter* multiplex PCR and the size of PCR products

Target	Primer	Gene	Size (Base pair)
Campylobacter	Forward 5'-TATACCGGTAAGGAGTGCTGGAG-3'	23S rRNA	650
	Reverse 5'-ATAAAAGACTATCGTCGCGTG-3'		
Campylobacter coli	Forward 5'-GTAAAACCAAAGCTTATCGTG-3'	*glyA* gene	126
	Reverse 5'-TCCAGCAATGTGTGCAATG-3'		
Campylobacter jejuni	Forward 5'-ACTTCTTTATTGCTTGCTGC-3'	*hipO* gene	323
	Reverse 5'-GCCACAACAAGTAAAGAAGC-3'		

3.2 Genetic relatedness of *Campylobacter jejuni* in Thai broiler houses

The second experiment consists of 4 phases including sample collection (phase I), *Campylobacter* isolation (phase II), *Campylobacter* confirmation and identification (phase III) and molecular characterization of *C. jejuni* by Comparative Genomic Fingerprint 40 (CGF40) (phase IV). Because direct plating of boot swab samples onto mCCDA and Preston agar gave the highest *Campylobacter* isolation rate in experiment I, boot swabs were obtained from 60 broiler houses belonging to 12 broiler farms (5 houses per farm) in experiment II. *Campylobacter* were isolated from those samples by direct plating method using mCCDA and Preston agar. Suspected *Campylobacter* colonies were confirmed and identified by multiplex PCR. *C. jejuni* isolated from broiler farms that had at least two *Campylobacter*-positive houses were further characterized by CGF40.

3.2.1 Sample collection

A total of 60 boot swab samples were obtained from 60 houses of 12 broiler farms with flocks at 4 – 5 weeks of age. Boot swabs were obtained as described earlier.

3.2.2 *Campylobacter* isolation

Campylobacter were isolated from boot swab samples by the direct plating method using mCCDA and Preston agar, then incubated under the same conditions as described in the first experiment.

3.2.3 *Campylobacter* confirmation and identification

Presumptive *Campylobacter* colonies were confirmed and identified by multiplex PCR as previously described.

3.2.3.1 Multiplex PCR

Multiplex PCR was performed to confirm and identify *Campylobacter* genus and species as described in the first experiment.

3.2.4 Molecular characterization of *C. jejuni* by Comparative Genomic Fingerprint (CGF40)

C. jejuni isolates from 8 farms, which had at least 2 houses positive to *C. jejuni*, were selected for molecular characterization. A total of 69 *C. jejuni* isolates from 31 houses were genotyped using CGF40. *C. jejuni* isolates were characterized by detecting 40 *Campylobacter* marker genes (Table 4). Eight sets of multiplex PCR specific to 40 genes were performed according to the previously published protocol (Taboada et al., 2012) with some modifications.

a. DNA extraction

For DNA extraction, *Campylobacter* isolates were sub-cultured onto non-selective blood agar (Columbia blood agar, CM 0331, Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and incubated under conditions as previously described. After incubation, *Campylobacter* colony was put into a microcentrifuge tube containing 100 µl of alkaline-PEG and homogenized by using vortex. Microcentrifuge tubes were heated in boiling water for 10 min, then they were immediately placed on ice and centrifuged at 18,928 ×g (13,000 rpm) for 5 min. After centrifugation, the supernatant was kept in a new microfuge tube and used as DNA template. DNA templates were kept frozen at -20°C.

b. DNA amplification

Forty genes were divided into 8 groups with 5 pairs of primer mixed in each group. Each reaction was carried out in a total volume of 25 µl containing 12.05 µl of nuclease-free water, 2.5 µl of 10x PCR buffer without $MgCl_2$ (Invitrogen, USA), 1.25 µl of 50 mM $MgCl_2$, 2 µl of 2.5 mM dNTPs (Sibenzyme, USA), 5 µl of 0.4 mM of each 10 primers mix, 0.2 µl Platinum Taq DNA polymerase (Invitrogen, USA) and 2 µl of DNA template. PCR conditions were performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C

for 30 sec except for the 7th primer set (Table 4) that 60°C was applied and extension at 65°C for 5 min with the final extension at 65°C for 20 min.

c. Gel electrophoresis

Six µl of PCR products were loaded into 1.5% agarose gel. Electrophoresis was carried out at 200 volts for 40 min. Afterward, agarose gel that contained PCR products was stained with ethidium bromide (EtBr) for 5 min and then washed by tap water for 15 min. The gel was analyzed by gel documentation system (Viber Loumat, France).

Table 4 The 8 multiplex PCR for CGF40 genes

C. jejuni marker genes	Amplicon size (bp)	*C. jejuni* marker genes	Amplicon size (bp)
Primer set 1		Primer set 2	
Cj0057	175	Cj0298c	198
Cj0860	282	Cj0728	296
Cj1431c	307	Cj0570	405
Cj0733	441	Cj0181	486
Cj1427c	613	Cj0483	612
Primer set 3		Primer set 4	
Cj0297c	300	Cj1550c	188
Cj1727c	369	Cj1329	307
Cj0264c	406	Cj0177	399
Cj0008	524	Cj1334	462
Cj1585c	630	Cj0566	558
Primer set 5		Primer set 6	
Cj0421c	100	Cj0755	101
Cj0033	206	Cj0736	205
Cj0486	301	Cj0967	301
Cj0569	399	Cj1141	413
Cj0625	498	Cj1136	510
Primer set 7		Primer set 8	
Cj1306c	151	Cj1294	160
Cj1552c	222	Cj1551c	241
Cj1439c	307	Cj0307	347
Cj1721c	415	Cj1324	440
Cj1679	529	Cj0035c	541

As described by Taboada et al. (2012)

3.3 Data analysis

Statistical analysis was conducted using IBM SPSS statistics 22 software. Descriptive analysis was used to describe the basic feature of the data. McNemar test (*p<0.05)* was used to compare *Campylobacter* isolation rates of each sample type and isolation method. CGF40 patterns were classified based on presence and absence of 40 genes. *Campylobacter* isolates that showed 100% similarity of presence and absence of genes were grouped as the same CGF40 pattern.

CHAPTER IV
RESULTS

4.1 Appropriate sample type and isolation method for *Campylobacter* detection from broiler farms

4.1.1 Occurrence of *Campylobacter* in different sample types

To determine the most suitable sample type, only *Campylobacter* isolation rates from direct plating method were compared. A total of 21 out of 60 houses (35%) were positive to *Campylobacter*. Boot swab samples gave the highest *Campylobacter* isolation rate at 26.7% (16/60) followed by cloacal swab samples (12/60; 20%) and fresh fecal samples (9/60; 15%) (Table 5). Broiler house was defined as *Campylobacter*-positive when at least one type of sample from that house was positive to *Campylobacter*.

Among *Campylobacter*-positive houses, ten broiler houses were positive to *Campylobacter* by one sample type, six houses were positive to *Campylobacter* by two sample types and five houses were positive to *Campylobacter* by all sample types. Among positive houses by one sample type, eight houses were positive by boot swabs, while the other two houses, one house was positive to *Campylobacter* by cloacal swab and another one was positive to *Campylobacter* by fresh fecal

sample. For six houses that positive by two types of sample, three houses were positive by boot swabs and cloacal swabs and the other three houses were positive by cloacal swabs and fresh fecal samples (Table 5).

Table 5 *Campylobacter*-positive houses by sample type using direct plating method

Positive house number	Sample type		
	Cloacal swabs	Boot swabs	Fresh fecal samples
1	-	+	-
2	-	+	-
3	+	-	+
4	+	+	+
5	-	+	-
6	+	+	-
7	+	+	+
8	+	+	+
9	-	+	-
10	-	-	+
11	-	+	-
12	+	+	-
13	+	+	-
14	+	-	+
15	+	-	-
16	-	+	-
17	+	+	+
18	-	+	-
19	-	+	-
20	+	+	+
21	+	-	+
Total	12[a]	16[a]	9[a]

(+) sign denotes *Campylobacter*-positive; (-) denotes *Campylobacter*-negative.

Superscript letter [(a)] denote not significant difference at *(p>0.05).*

4.1.2 Identification of appropriate isolation method for *Campylobacter* detection at the farm level

In order to compare the efficacy of isolation methods for *Campylobacter* detection at broiler farms, different types of isolation method including direct plating method and selective enrichment method were evaluated. As recommended by ISO standard method, a second medium should be used to increase *Campylobacter* isolation rate. However, the suitable type of second media was not clearly indicated in the protocol. Therefore, in the present study, the most commonly used medium, modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA), was compared with other three commercial selective media generally used for *Campylobacter* isolation including Preston agar, mKarmali agar and Campy-cefex agar. The significant difference of *Campylobacter* isolation rates was analyzed using McNemar test *($p<0.05$)*.

For cloacal swab samples tested by direct plating method on mCCDA, Preston agar, mKarmali agar and Campy-cefex agar, the results showed that the highest *Campylobacter* isolation rate was obtained by Preston agar (8/60; 13.3%), followed by mCCDA (7/60; 11.7%), Campy-cefex agar (6/60; 10%) and mKarmali agar (5/60; 8.3%) as shown in Table 6. However, no significant differences in

Campylobacter isolation rates among the tested media were observed. *P*-value of McNemar test for the difference in *Campylobacter* isolation rates from cloacal swab samples by each selective agar media is shown in Table 7.

Additionally, when combination of two select agar media was used, *Campylobacter* detection rates increased from 8.3% - 13.3% to 13.3% - 16.7% (Table 8). Since Preston agar gave the highest isolation rate followed by mCCDA, isolation rate obtained by using both Preston agar and mCCDA was considered. However, the isolation rate by using both Preston agar and mCCDA was not significantly different from the isolation rate by using Preston agar alone (Table 7). Likewise, no significant increase of *Campylobacter* isolation rates was observed when three or more types of media were used.

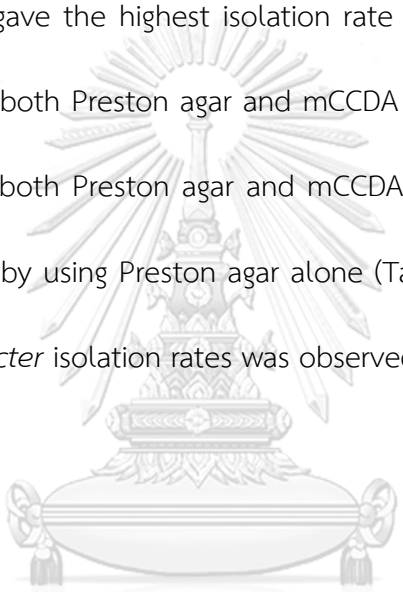


Table 6 *Campylobacter* isolated from cloacal swab samples by each type of media

Positive sample no.	Positive samples			
	mCCDA	mKarmali	Preston	Campy-cefex
1	-	-	+	+
2	+	-	+	+
3	-	-	+	-
4	+	-	+	-
5	-	-	+	+
6	+	-	-	-
7	+	+	+	+
8	+	+	+	+
9	-	-	-	+
10	+	+	-	-
11	-	+	-	-
12	+	+	+	-
12/60*	7/60[a] (11.6%)	5/60[a] (8.3%)	8/60[a] (13.33%)	6/60[a] (10%)

(+) sign denotes *Campylobacter*-positive; (-) sign denotes *Campylobacter*-negative.

*A total of 60 samples were examined in this study.

Superscript letter [(a)] denote not significant difference at *(p>0.05)*.

Table 7 McNemar test for the difference of *Campylobacter* isolation rates from cloacal swab samples by each type of media (*P*-value)

	mCCDA	mKarmali	Preston	Campy-cefex	mC+P*
mCCDA	*1*	-	-	-	-
mKarmali	*0.62*	*1*	-	-	-
Preston	*0.99*	*0.45*	*1*	-	-
Campy-cefex	*0.99*	*0.99*	*0.62*	*1*	-
mC+P*	*0.25*	*0.12*	*0.5*	*0.2*	*1*

Campylobacter isolation rates were significantly different when $p<0.05$.

*mC+P: Isolation rates by using both mCCDA and Preston agar.

Table 8 Isolation rates of *Campylobacter* from cloacal swab samples when different combinations of media were used

No. of media	Type of media	Positive sample (%) *
One type of media	mCCDA	7/60 (11.67%)
	mKarmali agar	5/60 (8.33%)
	Preston agar	**8/60 (13.33%)**
	Campy-cefex agar	6/60 (10%)
Two types of media	mCCDA+mKarmali agar	8/60 (13.33%)
	mCCDA+Preston agar	10/60 (16.67%)
	mCCDA+Campy-cefex agar	10/60 (16.67%)
	mKarmali agar+Preston agar	10/60 (16.67%)
	mKarmali agar+Campy-cefex agar	9/60 (15%)
	Preston agar+Campy-cefex agar	9/60 (15%)
Three types of media	mCCDA+mKarmali agar+Preston agar	11/60 (18.33%)
	mCCDA+mKarmali agar+Campy-cefex agar	11/60 (18.33%)
	mKarmali agar+Preston agar+Campy-cefex agar	11/60 (18.33%)
	Preston agar+Campy-cefex agar+mCCDA	11/60 (18.33%)
Four types of media	mCCDA + mKarmali agar + Preston agar+Campy-cefex agar	12/60 (20%)

*The number of positive samples/a total number of samples tested.

As shown in Table 9, *Campylobacter* isolation rates of fresh fecal samples by direct plating method on mCCDA or Preston agar was significantly higher than those on mKarmali agar and Campy-cefex agar. Although mCCDA yielded the highest isolation rate (9/60; 15%), it was not statistically different from Preston agar (8/60; 13.3%) *(p=0.99)*. *P*-value of McNemar test for the difference in *Campylobacter* isolation rates from fresh fecal samples by each selective agar media is shown in Table 10. Unlike cloacal swab samples, no increase in *Campylobacter* isolation rates was observed for fresh fecal samples when more than one types of media were used. In fact, the highest *Campylobacter* isolation rate was obtained by using mCCDA alone (Table 11). Among selective media used for isolation of *Campylobacter* from cloacal swab and fresh fecal samples, Preston agar and mCCDA provided the highest *Campylobacter* isolation rate for cloacal swab samples and fresh fecal samples, respectively.

Table 9 *Campylobacter* isolated from fresh fecal samples by each type of media

Positive sample no.	Positive samples			
	mCCDA	mKarmali	Preston	Campy-cefex
1	+	-	+	-
2	+	+	+	-
3	+	-	+	-
4	+	-	+	-
5	+	-	+	-
6	+	+	-	-
7	+	-	+	-
8	+	+	+	-
9	+	-	+	+
9/60*	9/60[a] (15%)	3/60[b] (5%)	8/60[a] (13.33%)	1/60[b] (1.67%)

(+) sign denotes *Campylobacter*-positive; (-) sign denotes *Campylobacter*-negative.

*A total of 60 samples were examined in this study.

Superscript letter [(a)] denote not significant difference at *(p>0.05).*

Superscript letter [(a,b)] denote significant difference at *(p<0.05).*

Table 10 McNemar test for the difference of *Campylobacter* isolation rates from fresh fecal samples by each type of media (*P*-value)

	mCCDA	mKarmali	Preston	Campy-cefex	mC+P*
mCCDA	*1*	-	-	-	-
mKarmali	*0.03*	*1*	-	-	-
Preston	*0.99*	*0.12*	*1*	-	-
Campy-cefex	*0.008*	*0.625*	*0.016*	*1*	-
mC+P*	*1*	*0.03*	*0.99*	*0.008*	*1*

Campylobacter isolation rates were significantly different when $p<0.05$.

*mC+P: Isolation rates by using both mCCDA and Preston agar.

Table 11 Isolation rates of *Campylobacter* from fresh fecal samples when different combinations of media were used

No. of media	Type of media	Positive sample (%) *
One type of media	**mCCDA**	**9/60 (15%)**
	mKarmali agar	3/60 (5%)
	Preston agar	8/60 (13.33%)
	Campy-cefex agar	1/60 (1.67%)
Two types of media	mCCDA+mKarmali agar	9/60 (15%)
	mCCDA+Preston agar	9/60 (15%)
	mCCDA+Campy-cefex agar	9/60 (15%)
	mKarmali agar+ Preston agar	9/60 (15%)
	mKarmali agar+Campy-cefex agar	4/60 (6.67%)
	Preston agar+Campy-cefex agar	8/60 (13.33%)
Three types of media	mCCDA+mKarmali agar+ Preston agar	9/60 (15%)
	mCCDA+mKarmali agar+Campy-cefex agar	9/60 (15%)
	Preston agar+ Campy-cefex agar+mCCDA	9/60 (15%)
	mKarmali agar+ Preston agar+ Campy-cefex agar	9/60 (15%)
Four types of media	mCCDA + mKarmali agar + Preston agar+Campy-cefex agar	9/60 (15%)

*The number of positive samples/a total number of samples tested.

Boot swab sampling method is mainly used for collecting samples from farm environment by wearing shoe cover and walking through broiler houses. Since *Campylobacter* in environment outside chicken body might be injured or weak, selective enrichment method may be useful and may help increase *Campylobacter* recovery rate from this type of sample.

Overall, our findings revealed that selective enrichment method provided lower isolation rates of *Campylobacter* from boot swab samples than direct plating method. As shown in Table 12, mCCDA could recover *Campylobacter* from 11 out of 60 samples (18.33%), which was significantly higher than mKarmali (*p=0.002*) and Campy-cefex (*p=0.01*) but was not significantly different from Preston agar (9/60; 15%) *(p= 0.7). P*-value of McNemar test for the difference in *Campylobacter* isolation rates from boot swab samples by each selective agar media is shown in Table 13. However, when a combination of mCCDA and Preston agar was used, *Campylobacter* isolation rates increased from 18.3% to 26.7%. According to our findings, direct plating method on both mCCDA and Preston agar provided the highest *Campylobacter* isolation rate for boot swab samples (Table 14). Therefore, this method should be used when the recovery of *Campylobacter* from farm samples is required.

Table 12 *Campylobacter* isolated from boot swab samples by direct plating and selective enrichment methods

Positive sample no.	Type of broth and media																			
	Direct method				Enrichment method															
					Bolton broth				Preston broth				Exeter broth				Blood-free Bolton broth			
	mC	mk	P	C	mC	mK	P	C	mC	mK	P	C	mC	mK	P	C	mC	mK	P	C
1	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
2	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	-
4	+	-	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
9	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
10	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
11	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	+	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
14		-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	+
15	-	-	-	-	-	-	+	+	+	-	+	+	+	-	+	-	+	-	-	-
16	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
18	+	-	+	-	-	-	-	-	+	+	-	+	-	-	+	-	-	-	+	-
19	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
20	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	11[a]	1[b]	9[a]	2[b]	4[ab]	1[b]	5[ab]	2[b]	7[ab]	6[ab]	5[ab]	5[ab]	3[ab]	3[ab]	5[ab]	4[ab]	1[b]	1[b]	1[b]	1[b]
Isolation rate	18%	1.7%	15%	3.3%	6.7%	1.7%	8.3%	3.3%	11%	10%	8.3%	8.3%	5%	5%	8.3%	6.7%	1.7%	1.7%	1.7%	1.7%

mC = mCCDA, mK = mKarmali, P = Preston, C = Campy-cefex (+) sign denotes *Campylobacter*-positive; (-) sign denotes *Campylobacter*-negative

*A total of 60 samples were examined for *Campylobacter*. Different superscript letters indicate significant difference ($p<0.05$)

Table 13 McNemar test for the difference of *Campylobacter* isolation rates from boot swab samples by each type of media (*P*-value)

	mCCDA	mKarmali	Preston	Campy-cefex	mC+P*
mCCDA	*1*	-	-	-	-
mKarmali	*0.002*	*1*	-	-	-
Preston	*0.7*	*0.02*	*1*	-	-
Campy-cefex	*0.01*	*0.99*	*0.01*	*1*	-
mC+P*	*0.06*	*0.00006*	*0.01*	*0.0001*	*1*

Campylobacter isolation rates were significantly different when $p<0.05$.

*mC+P: Isolation rates by using both mCCDA and Preston agar.

Table 14 Isolation rates of *Campylobacter* from boot swab samples when different combinations of media were used

The No. of Media	Type of Media	Direct plating method*	Selective enrichment method*			
			Bolton broth	Preston broth	Exeter broth	Blood-free Bolton broth
One type of media	mCCDA	11/60 (18.33%)	4/60 (6.67%)	7/60 (11.67%)	3/60 (5%)	1/60 (1.67%)
	mKarmali	1/60 (1.67%)	1/60 (1.67%)	6/60 (10%)	3/60 (5%)	1/60 (1.67%)
	Preston agar	9/60 (15%)	5/60 (8.33%)	5/60 (8.33%)	5/60 (8.33%)	1/60 (1.67%)
	Campy-cefex agar	2/60 (3.33%)	2/60 (3.33%)	5/60 (8.33%)	4/60 (6.67%)	1/60 (1.67%)
Two types of media	mCCDA+mKarmali	11/60 (18.33%)	4/60 (6.67%)	9/60 (15%)	3/60 (5%)	2/60 (3.33%)
	mCCDA+Preston agar	**16/60 (26.67%)**	7/60 (11.67%)	9/60 (15%)	6/60 (10%)	2/60 (3.33%)
	mCCDA+ Campy-cefex agar	12/60 (20%)	5/60 (8.33%)	8/60 (13.33%)	4/60 (6.67%)	2/60 (3.33%)
	mKarmali+Preston agar	10/60 (16.67%)	5/60 (8.33%)	8/60 (13.33%)	6/60 (10%)	2/60 (3.33%)
	mKarmali+ Campy-cefex agar	3/60 (5%)	2/60 (3.33%)	7/60 (11.67%)	4/60 (6.67%)	2/60 (3.33%)
	Preston agar+ Campy-cefex agar	9/60 (15%)	6/60 (10%)	7/60 (11.67%)	7/60 (11.67%)	2/60 (3.33%)
Three types of media	mCCDA+mKarmali+ Preston agar	16/60 (26.67%)	7/60 (11.67%)	9/60 (15%)	6/60 (10%)	3/60 (5%)
	mCCDA+mKarmali+ Campy-cefex agar	12/60 (20%)	5/60 (8.33%)	9/60 (15%)	4/60 (6.67%)	3/60 (5%)
	mCCDA+Preston agar+ Campy-cefex agar	16/60 (26.67%)	8/60 (13.33%)	8/60 (13.33%)	7/60 (11.67%)	3/60 (5%)
	mKarmali+ Preston agar+ Campy-cefex agar	10/60 (16.67%)	6/60 (10%)	8/60 (13.33%)	7/60 (11.67%)	3/60 (5%)
Four types of media	mCCDA+mKarmali+Preston agar+Campy-cefex agar	16/60 (26.67%)	8/60 (13.33%)	9/60 (15%)	7/60 (11.67%)	4/60 (6.67%)

*The number of positive samples/a total number of samples tested.

4.2 Genetic relatedness of *Campylobacter jejuni* in Thai broiler houses

A total of 69 *C. jejuni* isolates from 31 houses of 8 broiler farms that had at least 2 *C. jejuni* positive houses were selected for genetic characterization by CGF40 technique. Eight farms were classified as farm A-H. Among these farms, three farms (A, C and F) had five *C. jejuni* positive houses, while the other five farms contained both *C. jejuni* positive and negative houses (Table 15). Our results showed that 25 CGF40 patterns circulated in 31 houses of 8 broiler farms (Figure 11). Overall, *C. jejuni* isolates from different broiler farms had different CGF40 gene patterns, whereas *C. jejuni* from broiler houses in the same farm had quite similar CGF40 patterns. At least 2 CGF40 patterns were found in each broiler farm (Table 15). Farm A, B, G and H contained 2 CGF40 patterns, while farm C, D, E and F had 5, 6, 3 and 4 patterns, respectively. There were 10 houses that contained more than one CGF40 patterns. Besides, there is only one pattern, pattern no. 5, which was found in two broiler farms (C and D) in this study. Amplified fragment of the 8 multiplex PCRs detection for CGF40 are shown in Figures 3-10.

For the gene pattern, the number of genes that showed in each CGF40 pattern was 16-29 genes. Most patterns (>75%) contained 19-26 genes. The results revealed that a total of 7 genes including Cj0728, Cj0733, Cj0421c, Cj0625, Cj1294,

Cj0307 and Cj0035c appeared in all CGF40 patterns. In addition, twelve genes including Cj1431c, Cj0264c, Cj0008, Cj0033, Cj0569, Cj1141, Cj1136, Cj1552c, Cj1439c, Cj1721c, Cj1679 and Cj1551c were present only in few CGF40 patterns (Figure 11).

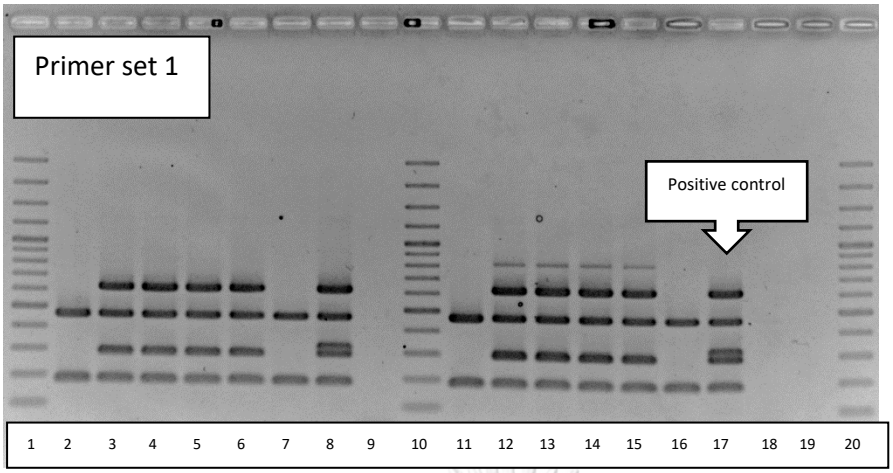


Figure 3 Multiplex PCR amplified fragments of CGF40 genes (primer set 1); Lanes 1,10,20: DNA size marker, Lanes 2,7,11 and 16: *C. jejuni* positive for genes Cj0733 and Cj0057; Lanes 3-6 and 12-15: *C. jejuni* positive for genes Cj1427c, Cj0733, Cj0860 and Cj0057; Lanes 8 and 17: standard strain NTCC11168 as positive control; Lanes 9 and 18: Negative control.

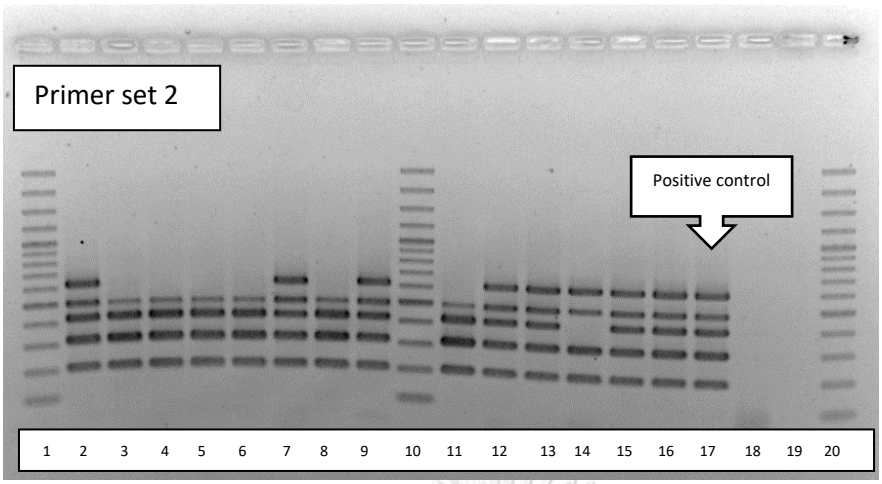


Figure 4 Multiplex PCR amplified fragments of CGF40 genes (primer set 2); Lanes 1,10,20: DNA size marker; Lanes 2,7,9,12,13,15 and 16: *C. jejuni* positive for genes Cj0298c, Cj0728, Cj0570, Cj0181 and Cj0483; Lanes 3-4,8,11: *C. jejuni* positive for genes Cj0298c, Cj0728, Cj0570 and Cj0181; Lane 14: *C. jejuni* positive for genes Cj0298c, Cj0728, Cj0181 and Cj0483; Lane 17: Standard strain NTCC11168 as positive control; Lane 18: Negative control.

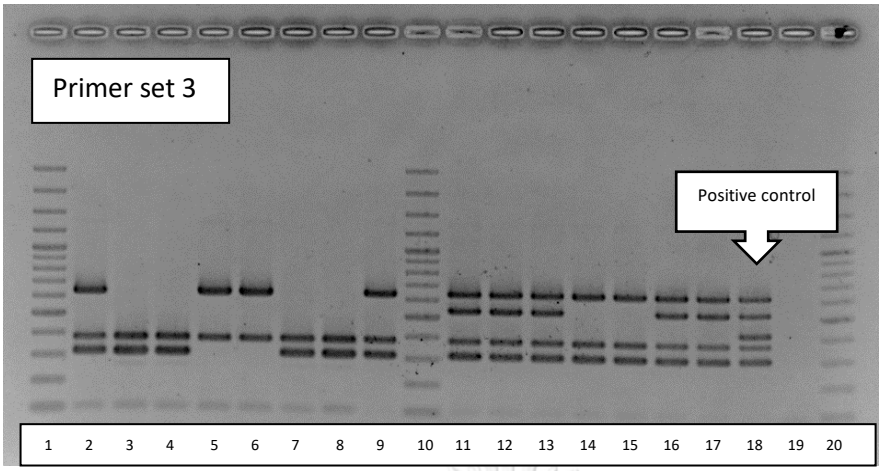


Figure 5 Multiplex PCR amplified fragments of CGF40 genes (primer set 3); Lanes 1,10,20: DNA size marker; Lanes 2,9,14 and 15: *C. jejuni* positive for genes Cj0297c, Cj0008 and Cj1585c; Lanes 3,4,7 and 8: *C. jejuni* positive for genes Cj0297c and Cj1727c; Lanes 5 and 6: *C. jejuni* positive for genes Cj1727c and Cj1585c; Lanes 11-13 and 16-17: *C. jejuni* positive for genes Cj0297c, Cj1727c, Cj0008 and Cj1585c; Lane 18: Standard strain NTCC11168 as positive control; Lane 19: Negative control.

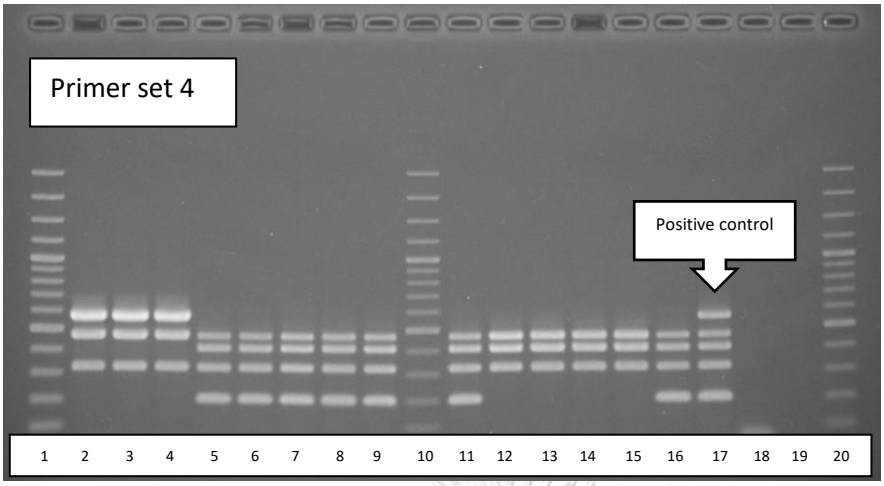


Figure 6 Multiplex PCR amplified fragments of CGF40 genes (primer set 4); Lanes 1,10,20: DNA size marker; Lanes 2-4: *C. jejuni* positive for genes Cj1329, Cj1334 and Cj0566; Lanes 5-11, 16: *C. jejuni* positive for genes Cj1550c, Cj1329, Cj0177 and Cj1334; Lanes 12-15: *C. jejuni* positive for genes Cj1329, Cj0177 and Cj1334; Lane 17: Standard strain NTCC11168 as positive control; Lane 18: Negative control.

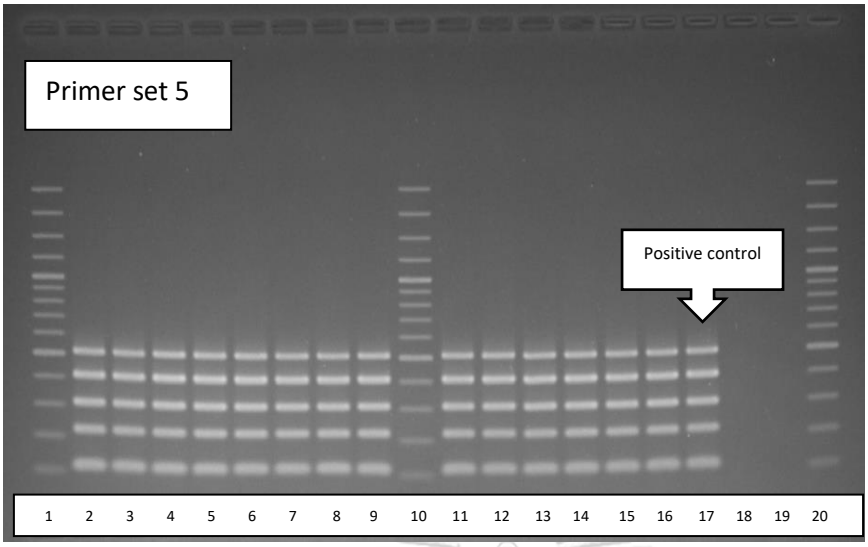


Figure 7 Multiplex PCR amplified fragments of CGF40 genes (primer set 5); Lanes 1,10,20: DNA size marker; Lanes 2-16: *C. jejuni* positive to gene Cj0421c, Cj0033, Cj0486, Cj0569 and Cj0625; Lane 17: Standard strain NTCC11168 as positive control; Lane 18: Negative control.

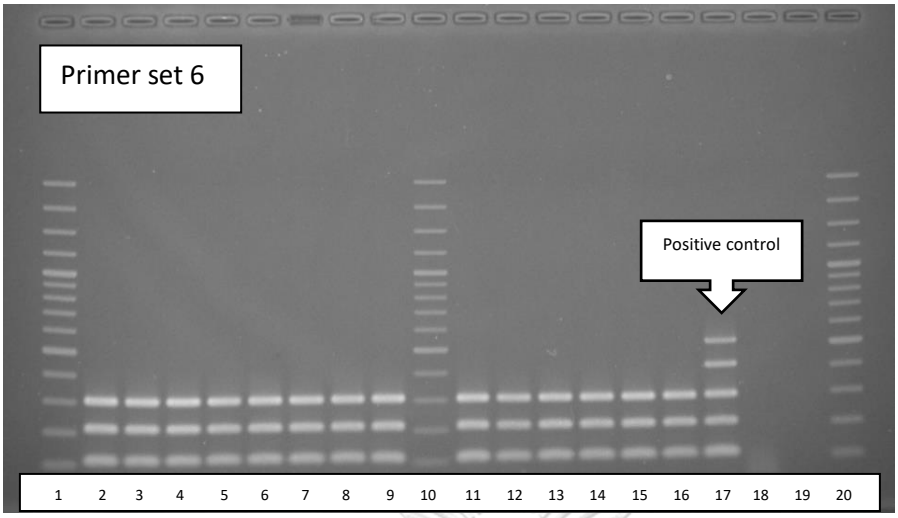


Figure 8 Multiplex PCR amplified fragments of CGF40 genes (primer set 6); Lanes 1,10,20: DNA size marker; Lanes 2-16: *C. jejuni* positive for genes Cj0755, Cj0736 and Cj0967; Lane 17: Standard strain NTCC11168 as positive; Lane 18: Negative control.

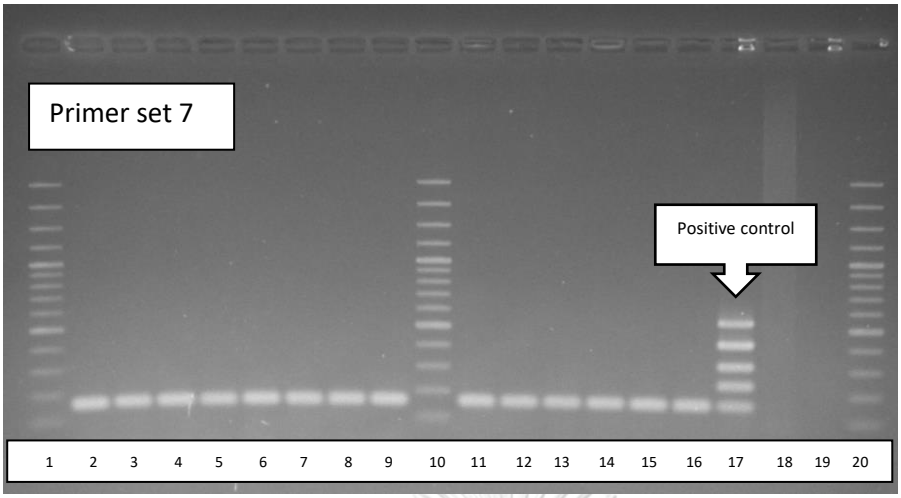


Figure 9 Multiplex PCR amplified fragments of CGF40 genes (primer set 7); Lanes 1,10,20: DNA size marker; Lanes 2-16: *C. jejuni* positive for genes Cj1306; Lanes 17: Standard strain NTCC11168 as positive control; Lane 18: Negative control.

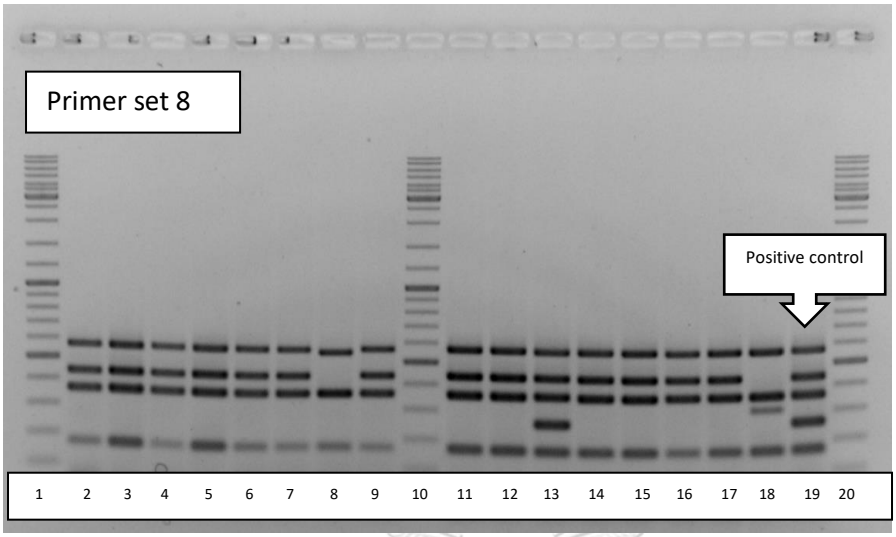


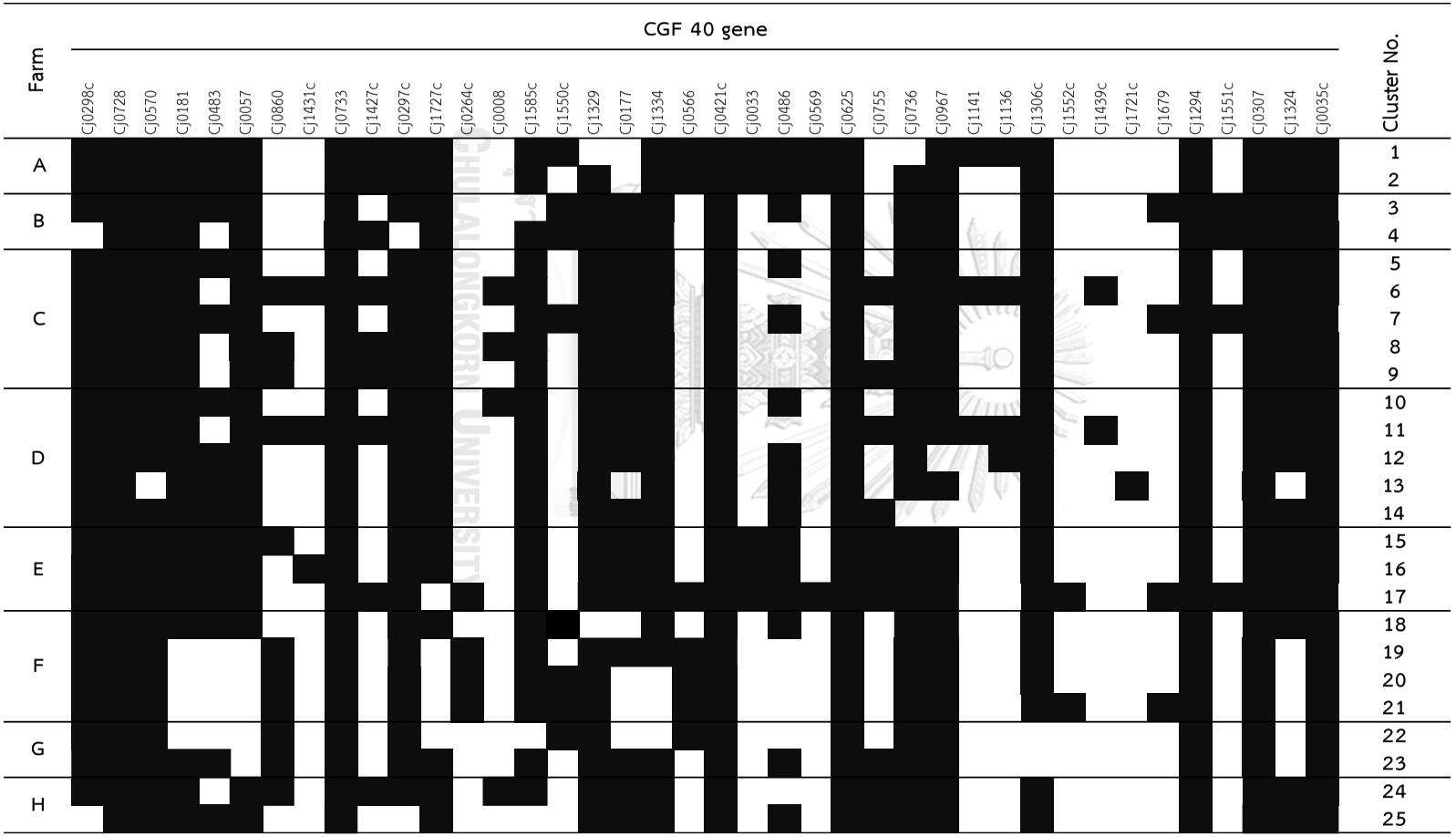
Figure 10 Multiplex PCR amplified fragments of CGF40 genes (primer set 8); Lanes 1,10,20: DNA size marker; Lanes 2-7, 9-12 and 14-17: *C. jejuni* positive for genes Cj0307, Cj1324 and Cj0035c; Lanes 8 and 18: *C. jejuni* positive for genes Cj0307 and Cj0035c; Lane 13: *C. jejuni* positive for genes Cj1294, Cj1551c, Cj0307, Cj1324 and Cj0035c; Lane 19: Standard strain NTCC11168 as positive control.

Table 15 CGF40 patterns of *C. jejuni* from 31 houses of 8 broiler farms

Farm	House No.	CGF40 pattern (No. of isolate)	No. of CGF40 patterns in the farms
A	1	1 (4)	2
	2	1 (2)	
	3	1 (3)	
	4	1 (4)	
	5	1,2 (4)	
B	1	3 (2)	2
	2	4 (2)	
	3	-	
	4	3 (2)	
	5	-	
C	1	5,6 (2)	5
	2	6 (2)	
	3	7 (2)	
	4	8 (2)	
	5	8,9 (2)	
D	1	10,11 (2)	6
	2	10,11 (2)	
	3	5,12 (2)	
	4	-	
	5	13,14 (2)	
E	1	15,16 (2)	3
	2	-	
	3	17 (2)	
	4	-	
	5	15 (2)	
F	1	18 (2)	4
	2	18,19 (2)	
	3	20,21 (2)	
	4	20 (2)	
	5	20 (2)	
G	1	-	2
	2	22 (2)	
	3	23 (2)	
	4	-	
	5	-	
H	1	24 (2)	2
	2	25 (2)	
	3	24 (2)	
	4	-	
	5	25 (2)	

(-) sign denotes *Campylobacter jejuni* negative house.

Figure 11 Twenty-five CGF40 patterns of 69 *C. jejuni* isolates from 31 houses of 8 broiler farms (black, present band; white, absent band.



CHAPTER V
DISCUSSIONS

5.1 Appropriate sample type and isolation method for *Campylobacter* detection from broiler farms

5.1.1 Occurrence of *Campylobacter* in different sample types

According to our study, prevalence of *Campylobacter* by cloacal swabs, fresh fecal samples and boot swab samples was 15-26.7%. Our findings found that boot swab samples gave *Campylobacter* isolation rates better than cloacal swab and fresh fecal samples. These findings are in line with the study of Vidal et al. (2013), which indicated that boot swab samples provided higher sensitivity for *Campylobacter* detection than cloacal swab samples and could be used as an alternative sampling method for *Campylobacter* detection in broiler farms. In addition, boot swab samples were found to be suitable for *Campylobacter* screening test by molecular methods such as loop-mediated isothermal amplification method (Romero et al., 2016) and quantitative real-time PCR (Sondergaard et al., 2014).

Boot swab samples provided the higher detection rates of *Campylobacter* in this study because it covered more area than other sample types. In addition, boot swab sampling was easy to perform without animal disturbance and this sampling

method has also been recommended for *Salmonella* detection in Thai broiler farms (Suddee and Raksakul, 2014). Therefore, in order to avoid the underestimation of *Campylobacter* detection rate, our study suggests that boot swab sampling method should be used for *Campylobacter* detection at broiler farms.

5.1.2 Identification of appropriate isolation method for *Campylobacter* detection at the farm level

Campylobacter culture techniques are based on the promotion of *Campylobacter* growth and inhibition of other microorganisms. Several types of media such as mCCDA, Preston agar, Kamali agar, Campy-cefex agar, Skirrow agar, Butzler agar, etc. were developed for *Campylobacter* isolation. Generally, supplements including antimicrobial agents, antifungal agents and essential nutrients were different among selective media. Since *Campylobacter* is less competitive than other microorganisms, antimicrobial agents and nutrients in selective media or selective enrichment broth are necessary for the promotion of *Campylobacter* growth. Antimicrobial agents were used to inhibit other microorganisms such as *E. coli, Proteus* and *Bacillus*. However, *E. coli* tends to be resistant to some antimicrobial agents used in *Campylobacter* selective media. Hence, several studies tried to adjust the ingredients of selective enrichment broth and media to improve

Campylobacter isolation ability (Chon et al., 2013a; Chon et al., 2013b; Chon et al., 2014a; Chon et al., 2014b).

Since *Campylobacter* are sensitive to oxygen, several supplements such as blood, charcoal, sodium pyruvate, ferrous sulphate and sodium metabisulphite have been included in *Campylobacter* selective media for detoxifying or preventing toxic oxygen (Bolton et al., 1984). Although supplements used in Preston agar and mCCDA are totally different, these two media had quite similar *Campylobacter* isolation rates in our study. Preston agar uses blood as an oxygen quencher, while mCCDA uses charcoal, sodium pyruvate and ferrous sulphate. In addition, Preston agar uses polymixin B, rifampicin, trimethoprim and cycloheximide to inhibit other microorganisms, whereas mCCDA uses cefoperazone and amphotericin B. It was found that polymixin B used in Preston agar was able to inhibit most of competing flora such as *E. coli*, *Proteus* spp., etc. and could enhance *Campylobacter* isolation rates (Chon et al., 2013b; Chon et al., 2013c).

According to our results, mKarmali or Campy-cefex gave *Campylobacter* isolation rates significantly lower than mCCDA or Preston agar. Although Campy-cefex and mCCDA use cefoperazone for inhibition of other microorganisms, Campy-cefex does not contain sodium deoxycholate, which is used in mCCDA. Sodium

deoxycholate can decrease the growth and interfere with the motility of *E. coli* and *Proteus* spp. (Bolton et al., 1984). Likewise, supplements used in mKarmali are quite similar to those used in mCCDA, but mKarmali lacks sodium deoxycholate and casein hydrolysate, which are added to promote the growth of *Campylobacter*.

According to ISO, *Campylobacter* recovery rates could be increased by using second media. Unfortunately, no selective second media is clearly stated in the protocol (ISO, 2006; ISO, 2017). In our study, four types of media were compared to identify the most suitable second media for *Campylobacter* isolation. Our results found that the highest *Campylobacter* isolation rate from cloacal swab samples was obtained by using Preston agar, while mCCDA was found to be more efficient than Preston agar for *Campylobacter* isolation from cloacal swabs in the study of Vaz et al. (2014). In this study, mCCDA gave the highest *Campylobacter* isolation rate for fresh fecal and boot swab samples. Differently, Preston agar and Campy-cefex agar was found to be more sensitive for *Campylobacter* isolation from fresh fecal samples than mCCDA in other studies (Oyarzabal et al., 2005; Potturi-Venkata et al., 2007).

Although mCCDA is the most commonly used media for *Campylobacter* isolation (Peterz, 1991; Engberg et al., 2000; Rodgers et al., 2012), the performance of

mCCDA to detect *Campylobacter* in some sample types is limited (Vaz et al., 2014), especially samples with high background microorganisms. Several studies found that Preston agar could give similar or better performance than mCCDA for *Campylobacter* isolation from chicken neck skin, feces and litter samples (Krause et al., 2006; Vaz et al., 2014).

Campy-cefex agar is recommended by USDA (2013) for *Campylobacter* detection from raw poultry products (USDA, 2013). Campy-cefex agar has been claimed to provide better *Campylobacter* isolation rates than mCCDA (Potturi-Venkata et al., 2007). However, our study showed that Campy-cefex agar and mKarmali yielded significantly lower *Campylobacter* isolation rates than mCCDA and Preston agar. Our study also revealed that culturing samples directly onto mKarmali agar showed extensive fungal growth, which might obscure *Campylobacter* colonies. These findings imply that Campy-cefex and mKarmali agar are not suitable for *Campylobacter* detection from broiler farm samples because selective supplements used in mKarmali and Campy-cefex might not be able to inhibit the growth of fungi and other microorganisms.

Although using second isolation media could increase the opportunity to recover *Campylobacter*, it is important to note that an appropriate second medium

must be selected. Generally, the second media should be different from the first media meaning that both charcoal-based and blood-based media should be used for *Campylobacter* isolation. Our findings recommended using mCCDA and Preston agar as the first and second media for *Campylobacter* isolation. The difference of supplements (antimicrobial agents and nutrients) used in Preston agar and mCCDA might be one of the reasons that Preston agar can increase isolation rate when it is used in combination with mCCDA.

Although the previous study suggested that enrichment method should be used for *Campylobacter* detection from boot swab samples (Vidal et al., 2013), our study found that direct plating method provided better *Campylobacter* isolation rates than selective enrichment method because selective enrichment broth used in the method could lead to the overgrowth of other microorganisms and obscure *Campylobacter* colonies. These findings agree with several studies, which reported that enrichment method did not improve *Campylobacter* isolation rates from broiler farm samples (Ingresa-Capaccioni et al., 2015a; Rodgers et al., 2017).

Bolton enrichment broth, which has been recommended by ISO (2006), was less sensitive and not suitable for detection of *Campylobacter* from high background samples such as raw meat products, feces, etc. (Ugarte-Ruiz et al., 2012; Gharst et al.,

2013). Likewise, blood-free Bolton broth, which has been recommended by USDA (2013), also provided less sensitivity for *Campylobacter* isolation from boot swab samples in this study. Vidal et al. (2013) reported that Exeter broth gave better isolation rate for *Campylobacter* detection from boot swab samples, while our study revealed that Preston broth provided a better *Campylobacter* isolation rate than the other selective enrichment broths tested. The new revision of ISO standard method for *Campylobacter* detection (ISO, 2017) indicated that Preston broth should be used for *Campylobacter* isolation from samples with high background microorganisms such as samples from farms. High concentration of polymixin B in Preston broth was found to be the reason to enhance *Campylobacter* isolation rates because it can effectively inhibit gram-negative bacteria such as extended-spectrum beta-lactamase (ESBL) producing *E. coli*, *Proteus* spp., etc. (Chon et al., 2013b; Chon et al., 2013c).

5.2 Genetic relatedness of *Campylobacter jejuni* in Thai broiler houses

Campylobacter detection in broiler farms has not been regularly conducted, even though *Campylobacter* could be detected at the farm level and found to be related to *Campylobacter* along the food chain (Prachantasena et al., 2016). Member states of the European Union detected *Campylobacter* prevalence by collecting samples from only one house per farm (Kapperud et al., 1993; Evans and Sayers, 2000; Thakur et al., 2013). However, our study revealed that *Campylobacter*-positive and negative houses could be found in the same farm. Similar findings were also reported in other studies (Guerin et al., 2007; Vidal et al., 2016). Hence, *Campylobacter* detection from only one house could attribute to an underestimation of the prevalence of *Campylobacter*. If possible, *Campylobacter* should be detected from a few broiler houses in the same farm to accurately identify *Campylobacter* status of the farm. Thus, sorting flock prior to slaughtering processes can be properly conducted, which may help reduce or prevent *Campylobacter* contamination during processing steps (Bronzwaer et al., 2009; Berghaus et al., 2013; Haas et al., 2017).

In terms of genetic relatedness of *C. jejuni*, our results indicated that genetic profiles of *C. jejuni* among broiler houses within the same farm were quite similar.

On, while each farm seemed to have individual and unique genetic pattern by CGF40 technique. Similar to our finding, Vidal et al. (2016) also found a high genetic diversity of *C. jejuni* among broiler farms, but low genetic variation of *C. jejuni* among broiler houses in the same broiler farm.

The results obtained from CGF40 technique was consistent with those of multilocus sequence typing (MLST) technique, but CGF40 is easier to perform, low cost and less time consuming (Taboada et al., 2008; Clark et al., 2012; Taboada et al., 2013; Thepault et al., 2018). CGF40 technique examines the presence and absence of 40 selected genes as described in the previous study (Taboada et al., 2008), while MLST is *Campylobacter* genetic characterization technique by determining sequencing of nucleotide bases of seven house-keeping genes. Although CGF40 technique has discriminatory power similar to that of MLST, the results of CGF40 technique could not be compared between studies due to the lack of online database and this technique was currently developed for genetic characterization of *C. jejuni* only.

CONCLUSION AND SUGGESTION

In conclusion, boot swab sampling method provided better *Campylobacter* isolation rate than cloacal swab and fresh fecal samples. Hence, boot swabs should be used for *Campylobacter* detection at broiler farms. For *Campylobacter* isolation, direct plating of boot swab samples onto both mCCDA and Preston agar is recommended because it provided the highest *Campylobacter* isolation rate for samples from broiler farms. In addition, since *Campylobacter* flock status might be different among houses within the same farm, *Campylobacter* detection from a few or more houses is recommended in order to obtain more reliable *Campylobacter* flock status. However, because genetic profile of *C. jejuni* among isolated from different broiler houses in the same farm was quite similar, if it is required, molecular epidemiology of *C. jejuni* can be examined from one of *Campylobacter*-positive houses in the same farm.

Further study will be required to understand molecular epidemiology of *C. jejuni* isolated from different types of samples in broiler farms and the impact of sampling method on genetic profiles of *C. jejuni*. Genetic profiles of *C. jejuni* isolated from boot swab sampling method may be different from those of *C. jejuni* isolated from cloacal swabs or cecal content, which were inside chicken body. Therefore, in order to support that boot swab sampling method can be used instead of cloacal swab samples for molecular epidemiology of *Campylobacter* in broiler farms, genetic

examination of *C. jejuni* from other sample types such as cloacal swabs should be compared with *C. jejuni* isolated from boot swab samples.

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APPENDIX

Appendix A

Culture media used for *Campylobacter* isolation

1. **Modified Charcoal-Cefoperazone-Deoxycholate (mCCDA)** was made by *Campylobacter* agar base (CM0739; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and CCDA supplement (SR0155; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) 1 vial/500ml.

2. **Modified Karmali (mKarmali)** agar was made by *Campylobacter* Agar Base (Karmali) (CM0935; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and modified karmali selective supplement (SR0205; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) 1 vial/500ml.

3. **Preston agar** was made by *Campylobacter* agar base (CM0689; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom, *Campylobacter* growth supplement (SR0232; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and *Campylobacter* selective supplement (Preston) (SR0117; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) 1 vial/500ml and sheep blood 50 ml/liter.

4. **Campy-cefex agar** was made by Brucella medium base 43 g/liter (CM0169; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), ferrous sulfate 0.5 g/ liter, sodium bisulfite 0.2 g/ liter, sodium pyruvate 0.5 g/ liter, cefoperazone 33 mg/liter, cycloheximide 0.2 g/liter and sheep blood 50 ml/liter.

5. **Bolton broth** was made by Bolton broth (CM0983; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), Bolton broth selective supplement (SR0183; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) 1 vial/ 500 ml and sheep blood 50 ml/liter.

6. **Preston broth** was made by Nutrient broth No. 2 (CM0067; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), *Campylobacter* growth supplement (SR0232; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) 1 vial/ 500 ml, *Campylobacter* selective supplement (SR0117; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) 1 vial/500 ml and sheep blood 50 ml/liter.

7. **Exeter broth** was made by Nutrient broth No. 2 (CM0067; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), sodium pyruvate 250 mg/liter, ferrous sulphate 250 mg/liter, sodium metabisulphite 250 mg/liter, trimethoprim, 10 mg/liter, rifampicin, 5 mg/ liter, polymyxin B, 2,500 IU/ liter, cefoperazone, 15 mg/ liter amphotericin B, 2 mg/liter and sheep blood 50 ml/liter.

8. **Blood-free Bolton broth** was made by Bolton broth (CM0983; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and added Bolton broth selective supplement (SR0183; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) 2 vials/500 ml.

Appendix B

Chemical substances

1. 50X Tris acetate EDTA (TAE)

Typical formula	(gm/liter)
Tris	220
Gracial acetic acid	57.1 ml
EDTA (0.5 Molar) pH 8	100 ml

Adding deionized water to reach 1,000 ml

2. 1X TAE buffer

Typical formula	(ml/liter)
50X TAE	20
Deionized water	980

3. Catalase solution

Typical formula	
Hydrogen peroxide 30%	10
Deionized water	90

4. 3.5% Ninhydrin solution

Typical formula

Ninhydrin	35 gm
Acetone	50 ml
Butanol	50 ml

5. Oxidase solution

Typical formula

Ascorbic acid	0.03 gm
Tetramethyl-p-Phenylendiamine Dihydrochloride	0.03 gm
Sterile water	30 ml

6. 1% Hippurate solution

Typical formula

Natriumhippurate	1 gm
PBS	99 ml

Appendix C

Campylobacter colony morphology

Campylobacter colonies on charcoal-based media (mCCDA and mKarmali) have smooth edge, round and flat with grayish metallic sheen, while colonies on blood-based media (Preston agar and Campy-cefex agar) have a smooth edge, round, flat and slightly pink.

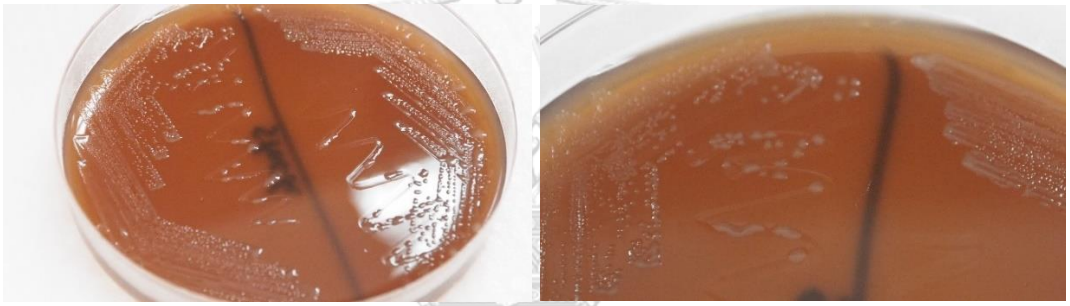

Figure C-1. *Campylobacter* colonies morphology on blood-based media

Figure C-2. *Campylobacter* colonies morphology on charcoal-based media

Appendix D

Biochemical tests

1. Catalase test

Suspected *Campylobacter* colonies were applied on glasses slide and one drop of 3% H_2O_2 was added onto *Campylobacter* colonies. Bubble gas that was immediately present indicated positive result.

2. Oxidase test

Suspected *Campylobacter* colonies were put on filter paper (Whatman®, Germany). After that, oxidase solution (Ascorbic acid, 3 mg/ml; N,N,N´,N´- Tetramethyl-p-Phenylanesdiamine Dihydrochloride, 3 mg/ml and sterile water) was dropped onto the colonies. Dark blue or purple color observed within 10 seconds indicated positive result.

3. Hippurate hydrolysis test

A loopful of suspected *Campylobacter* colonies was put into glass tubes containing 400 µl of 1% sodium Hippurate solution (sodium hippurate, 1 g; Phosphate buffer saline, 99 ml). After that, the glass tubes were incubated at 37°C for 4 hours. Subsequently, 3.5% ninhydrin solution (Ninhydrin, 3.5 g; Acetone 50 ml and Butanol 50 ml) was added into the glass tube and incubated at 37°C for 10 min.

Purple color indicated a positive result, while grey color indicated negative result. *C. jejuni* is recognized as hippurate hydrolysis positive, whereas *Campylobacter coli* and *Campylobacter lari* are considered as hippurate hydrolysis negative.

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