การปนเปื้อนของเชื้ออาร์โคแบคเตอร์ในโรงแปรรูปไก่เนื้อสองแห่งในประเทศไทย

นางสาวลักษณ์ หาญกล้า

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

ARCOBACTER CONTAMINATION IN TWO POULTRY PROCESSING PLANTS IN THAILAND

Miss Luck Hankla

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Public Health

Department of Veterinary Public Health

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2015

Thesis Title	*ARCOBACTER* CONTAMINATION IN TWO POULTRY PROCESSING PLANTS IN THAILAND
By	Miss Luck Hankla
Field of Study	Veterinary Public Health
Thesis Advisor	Taradon Luangtongkum, D.V.M., Ph.D.

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ลักษณ์ หาญกล้า : การปนเปื้อนของเชื้ออาร์โคแบคเตอร์ในโรงแปรรูปไก่เนื้อสองแห่งในประเทศไทย (*ARCOBACTER* CONTAMINATION IN TWO POULTRY PROCESSING PLANTS IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ธราดล เหลืองทองคำ, 68 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจหาอุบัติการณ์และลักษณะทางพันธุกรรมของเชื้ออาร์โคแบคเตอร์จากขั้นตอนต่าง ๆ ภายในโรงแปรรูปและเพื่อตรวจสอบแหล่งที่มาของการปนเปื้อนเชื้ออาร์โคแบคเตอร์ในเนื้อไก่ ในการศึกษาครั้งนี้ทำการเก็บตัวอย่างทั้งหมด 388 ตัวอย่าง จากโรงแปรรูปเนื้อไก่ 2 แห่งในประเทศไทย (โรงแปรรูป A และ โรงแปรรูป B) โดยแบ่งเป็นตัวอย่างที่มาจากไก่ 152 ตัวอย่าง และตัวอย่างที่มาจากสิ่งแวดล้อมในโรงแปรรูป 236 ตัวอย่าง การเพาะแยกเชื้ออาร์โคแบคเตอร์จะใช้วิธี membrane filtration technique ในขณะที่การวิเคราะห์สายพันธุ์และการศึกษาลักษณะทางพันธุกรรมของเชื้อที่แยกได้จะใช้เทคนิค multiplex polymerase chain reaction และ Repetitive element sequence based PCR (rep-PCR) ด้วยไพรเมอร์ GTG_5 ตามลำดับ ผลการศึกษาพบว่าอุบัติการณ์ของเชื้ออาร์โคแบคเตอร์ในตัวอย่างจากโรงแปรรูป A อยู่ที่ 67% สำหรับการเก็บตัวอย่างครั้งที่ 1 และ 74% สำหรับการเก็บตัวอย่างครั้งที่ 2 ในขณะที่อุบัติการณ์การปนเปื้อนของเชื้ออาร์โคแบคเตอร์ในการเก็บตัวอย่างครั้งที่ 1 และครั้งที่ 2 ของโรงแปรรูป B อยู่ที่ 53.2% เท่ากัน การศึกษาครั้งนี้ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของอุบัติการณ์การปนเปื้อนของเชื้ออาร์โคแบคเตอร์ในโรงแปรรูปทั้งสองแห่ง สายพันธุ์ของเชื้ออาร์โคแบคเตอร์ที่พบมากที่สุดในการศึกษาครั้งนี้ ได้แก่ *Arcobacter butzleri* ผลการศึกษาลักษณะทางพันธุกรรมของเชื้ออาร์โคแบคเตอร์ด้วยเทคนิค rep-PCR พบว่าเชื้ออาร์โคแบคเตอร์ที่ปนเปื้อนในโรงแปรรูปทั้งสองแห่งมีความหลากหลายทางพันธุกรรมค่อนข้างมาก การที่ลักษณะทางพันธุกรรมของเชื้อที่แยกได้จากตัวอย่างสิ่งแวดล้อมมีความคล้ายคลึงกับลักษณะทางพันธุกรรมของเชื้อที่แยกได้จากไก่ อาจเนื่องมาจากการสัมผัสระหว่างซากไก่และสิ่งแวดล้อมในขั้นตอนต่าง ๆ ของกระบวนการเชือด ถึงแม้การเก็บตัวอย่างทั้ง 2 ครั้งจะห่างกันหลายสัปดาห์ แต่รูปแบบของ rep-PCR จากตัวอย่างในครั้งที่ 2 พบว่ามีความคล้ายคลึงกับรูปแบบของ rep-PCR จากตัวอย่างที่เก็บในครั้งที่ 1 ผลการศึกษาดังกล่าวแสดงให้เห็นว่าเชื้ออาร์โคแบคเตอร์ยังคงหมุนเวียนอยู่ภายในโรงแปรรูป เนื่องจากแหล่งที่มาของการปนเปื้อนของเชื้ออาร์โคแบคเตอร์สู่เนื้อไก่ภายในโรงแปรรูปยังไม่เป็นที่ทราบแน่ชัด การศึกษาถึงแหล่งที่มาของการปนเปื้อนดังกล่าวจึงยังคงมีความจำเป็น ทั้งนี้เพื่อช่วยให้การลดอุบัติการณ์ของเชื้ออาร์โคแบคเตอร์ในเนื้อไก่สามารถดำเนินการได้อย่างมีประสิทธิภาพ

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

5575316631 : MAJOR VETERINARY PUBLIC HEALTH
KEYWORDS: ARCOBACTER / CHICKEN / CONTAMINATION / PROCESSING PLANTS

LUCK HANKLA: *ARCOBACTER* CONTAMINATION IN TWO POULTRY PROCESSING PLANTS IN THAILAND. ADVISOR: TARADON LUANGTONGKUM, D.V.M., Ph.D., 68 pp.

This study aimed to determine the occurrence and genetic profiles of *Arcobacter* spp. from various slaughtering stages and to investigate the potential source of carcass contamination. A total of 388 samples consisting of chicken related samples (n=152) and environmental samples (n=236) were collected from two poultry processing plants (Plant A and Plant B) in Thailand. *Arcobacter* was isolated using the membrane filtration technique and identified to species level using a multiplex polymerase chain reaction method. Isolates were further genotyped by Repetitive element sequence based PCR (rep-PCR) using GTG_5 primers. The occurrence of *Arcobacter* in plant A was 67% and 74% on the first and the second sampling days, respectively. In plant B, the occurrence was 53% in both sampling days. No significant difference between the occurrence of *Arcobacter* in both plants was observed. *Arcobacter butzleri* was the predominant species observed in this study. Although cluster analysis of rep-PCR patterns revealed the high degree of genetic diversity of *Arcobacter* in Thai poultry processing plant, several *Arcobacter* genotypes present in the slaughterhouse environment were detected in chickens. This finding was likely due to the cross-contamination between slaughterhouse environment and chicken products along the processing line. Interestingly, even though the two sampling days were several weeks apart, some of *Arcobacter* isolates from the second sampling day had similar rep-PCR patterns with the isolates from the first sampling day. This finding indicated that some *Arcobacter* genotypes may be able to persist and circulate in the slaughterhouse environment. Since the exact route of poultry carcass contamination still remains unclear, further studies are required to investigate the source of *Arcobacter* contamination in order to effectively reduce the occurrence of this emerging foodborne pathogen in chicken carcasses.

Department: Veterinary Public Health Student's Signature .....
Field of Study: Veterinary Public Health Advisor's Signature .....
Academic Year: 2015

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Taradon Luangtongkum, for his academic guidance, encouragement, and patience throughout my research. This thesis would not have been accomplished without the support and dedication of my advisor. My sincere appreciation is extended to Dr. Nipa Chokesajjawatee and all staffs at Food Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC) for providing assistance and the opportunity to learn and complete my genotyping work at BIOTEC laboratory. My deepest thanks also go to all the committee members for giving me the precious advices. Moreover, I would like to take this opportunity to express my thanks to all staffs of the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University especially my dear colleagues in Campylobacter and Arcobacter Research Laboratory. I want to express my deep appreciation for all their help and support.

At last, I would like to dedicate this thesis to my family who always support me and stand by my side. I couldn't have done it without the love and support of my family.

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LIST OF ABBREVIATION

BB	boneless breast
BIL	bone in leg
bp	base pair(s)
BPW	buffered peptone water
ºC	degree (s) Celsius
CAT	cefoperazone-amphotericin B-teicoplanin
DNA	deoxyribonucleic acid(s)
h	hour (s)
I/O washing	Inside/Outside washing
mCCDA	modified Charcoal Cefoperazone Deoxycholate Agar
min	minute (s)
ml	milliliter (s)
mm	millimeter (s)
n	number
PEG	polyethylene glycol
Rep-PCR	Repetitive sequence-based polymerase chain reaction
sec	second (s)
spp.	species
TE	Tris-EDTA
TBE	Tris-Borate EDTA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
µl	microliter (s)
UV	Ultra violet
V	volt (s)

CHAPTER I
INTRODUCTION

Arcobacter is a gram-negative, motile, spiral-shaped bacterium belonging to the genus *Campylobactereaceae*. Although *Arcobacter* is closely related to *Campylobacter* spp., it can be differentiated from *Campylobacter* spp. by its ability to grow in the presence of air and at lower temperature (Vandamme et al., 1991). *Arcobacter* has been considered as a new emerging foodborne pathogen. In addition, it is also received an increasing attention to public health as a zoonotic agent (Vindigni et al., 2007). In 2002, the International Commission on Microbiological Specifications for Foods (ICMSF) classified *Arcobacter* spp. as an emerging pathogen which poses a serious hazard to human health. At present, *Arcobacter butzleri*, *Arcobacter cryaerophillus*, and *Arcobacter skirrowii* have been associated with human diseases and have been isolated most frequently from human enteritis cases. Symptoms of *Arcobacter* infection in human are similar to those of *Campylobacter* which are persistent diarrhea, abdominal pain, nausea, vomiting and fever. In addition, septicemia can occur sometimes (Vandenberg et al., 2004). Among *Arcobacter* species, *Arcobacter butzleri* is the most common species that causes diarrhea in human (Rivas et al., 2004). *Arcobacter* can be isolated from water, animals and foods of animal origin including chicken, beef, pork, and seafood. High prevalence of *Arcobacter* was reported in chicken meat (Rahimi, 2014). Handling of raw or consumption of undercooked meat or contaminated water is considered as a main source of *Arcobacter* infection in humans (Ho et al., 2006).

Although the high prevalence of *Arcobacter* in chicken meat has been reported in several studies worldwide (Lee et al., 2010; Rahimi, 2014; Zacharow et al., 2015), the exact route of contamination is still unclear. Many researchers suggested that the contamination of *Arcobacter* in chicken carcasses may take place at the slaughterhouse level along the processing line (Houf et al., 2003; Gude et al., 2005;

Ho et al., 2008). It was previously reported that *Arcobacter* was isolated from water used in processing plants. In addition, some studies found that similar *Arcobacter* genotypes were detected in both broiler carcasses and slaughter equipment at different slaughtering stages indicating that *Arcobacter* can be present in the slaughterhouse environment and cross contaminate to chicken meat during processing (Houf et al., 2002b; Houf et al., 2003; Son et al., 2007). Unlike *Campylobacter*, which is a natural colonizer of chicken intestinal tract, *Arcobacter* was rarely isolated from GI tract of chicken (Gude et al., 2005). Due to the lack of information on *Arcobacter* in poultry processing plants, it is difficult to explain why *Arcobacter* contamination rates in chicken carcasses were substantially high. Therefore, the aims of the present study were to determine the genetic relatedness of *Arcobacter* strains isolated from different processing steps and to investigate the potential source of *Arcobacter* contamination in poultry processing plants. The information obtained from this study will reveal the possible route of *Arcobacter* contamination in chicken carcasses in Thai poultry processing plants and provide knowledge that can be used for developing *Arcobacter* control strategies that can help reduce *Arcobacter* contamination in chicken carcasses in the future.

CHAPTER II
LITERATURE REVIEW

2.1. *Arcobacter* species

Arcobacter is a gram-negative bacterium which belongs to the family *Campylobacteriaceae*. *Arcobacter* differs from *Campylobacter* by its ability to grow at lower temperature ranging between 15-37 $^{\circ}$C and aerotolerant feature (Vandamme et al., 1991). *Arcobacter* can motile with a single or bipolar unsheathed flagellum at the end. The estimate size of *Arcobacter* is around 0.2–0.9 mm wide and 0.5–3 mm long. *Arcobacter* yields positive results to oxidase, catalase, and nitrate reduction tests. The genus *Arcobacter* is composed of 19 species including *A. butzleri, A. cryaerophilus*, *A. skirrowii, A. nitrofigilis, A. cibarius, A. halophilus, A. mytili*, *A. thereius, A. marinus, A. trophiarum, A. defluvii, A. molluscorum, A. bivalviorum, A. venerupis, A. ellisii, A. cloacae, A. suis, A. ebronensis*, and *A. aquimarinus*. However, only *A. butzleri, A. cryaerophilus*, *A. skirrowii,* and *A. cibarius* are associated with human diseases and animal infections (Vandamme et al., 1992; Donachie et al., 2005; Houf et al., 2005; Collado et al., 2009; Houf et al., 2009; Kim et al., 2010; Collado et al., 2011; De Smet et al., 2011b; Figueras et al., 2011a; Figueras et al., 2011b; Levican et al., 2012; Levican et al., 2013; Levican et al., 2015).

2.2. *Arcobacter* in human and the transmission route

In 2002, the International Commission on Microbiological Specifications for Foods (ICMSF) classified *Arcobacter* as an emerging foodborne pathogen (Mandisodza et al., 2012; Lappi et al., 2013). *Arcobacter* appears to have the same pathogenic properties as *Campylobacter*. The most common symptoms of *Arcobacter* infection are acute watery diarrhea, abdominal cramp, fever, and nausea. Sometimes, septicemia can also occur (Ho et al., 2006). Although the exact route of *Arcobacter*

transmission to human remains to be determined, several researchers suggested that human can become infected with *Arcobacter* due to handling or consumption of contaminated water and undercooked food especially chicken meat (Jacob et al., 1993; Lappi et al., 2013). Previous studies revealed that consumption of undercooked or contaminated chicken meat are the most likely source of *Arcobacter* infection in human (Scullion et al., 2006). Moreover, *Arcobacter* is not only found in foods of animal origin, but it is also isolated from various water sources including river water and drinking water (Ho et al., 2006). Some studies showed that *Arcobacter* species could be found in vegetables, such as lettuce (González and Ferrús, 2011). In addition, *Arcobacter* infection in human can be acquired through contact with pets such as cats and dogs that harbor *Arcobacter* in their oral cavity (Houf et al., 2008; Fera et al., 2009).

2.3. *Arcobacter* in animals and foods of animal origin

Arcobacter can be isolated from various animals such as pigs (Scanlon et al., 2013), cattle (Piva et al., 2013), poultry (Adesiji et al., 2011), shellfish (Levican et al., 2012) and wildlife animals (Wesley and Schroeder-Tucker, 2011). *Arcobacter* has been detected in several foods of animal origin such as beef, pork and poultry, which higher prevalence of this organism has been reported in chicken meat (Gonzalez et al., 2010; Lee et al., 2010). *A. buzleri* was the predominant species isolated from retail meat (Atabay et al., 2003). Although *Arcobacter* was rarely detected in the intestinal content of chicken, it was mainly found in stool of pigs and cattle (Wesley et al., 2000; De Smet et al., 2011a). In addition, *Arcobacter* can be isolated from raw milk and milk products (Serraino et al., 2013).

2.4. *Arcobacter* in slaughterhouses

The origin of *Arcobacter* contamination in poultry meat may occur at the slaughterhouse level (Gude et al., 2005). However, the source of *Arcobacter* in

slaughterhouse and the route of cross-contamination during meat processing are not well established. *Arcobacter* could be found in chicken carcasses along the slaughter processing line at different slaughtering processes such as before and after scalding, evisceration, and chilling (Son et al., 2007). One study indicated that *Arcobacter* could be detected in live birds and slaughter equipment before the onset of slaughtering (Houf et al., 2003). Several authors suggested that slaughter equipment should not be the main route of *Arcobacter* contamination during poultry slaughtering (Houf et al., 2002b; Houf et al., 2003; Ho et al., 2008). Since *Arcobacter* was found in chicken feces, it was suggested that poultry might be a natural reservoir of *Arcobacter* (Atabay et al., 2006; Ho et al., 2008). In addition, some studies reported that water used in poultry processing plants could also be the source of *Arcobacter* contamination in chicken carcasses (Atabay and Corry, 1997; Gude et al., 2005; Van Driessche and Houf, 2007).

Several studies have shown that *Arcobacter* can grow or survive in the wide range of temperature (5-37 $^{\circ}$C) by forming biofilm under chilled conditions (Kjeldgaard et al., 2009; Ferreira et al., 2013). To date, only limited information on *Arcobacter* in the slaughterhouse environment is available and the exact routes of *Arcobacter* contamination in chicken carcass are still unclear.

2.5. *Arcobacter* isolation and identification

Arcobacter has been isolated by selective enrichment method using *Arcobacter* enrichment broth added with cefoperazone, amphotericin and teicoplanin (CAT) supplement. This method provided suitable growth conditions for *Arcobacter* and suppressed the growth of competitive microorganisms (Atabay and Corry, 1998). A membrane filtration technique on modified charcoal cefoperazone deoxycholate agar (mCCDA) supplemented with antibiotics was also commonly used for *Arcobacter* isolation due to its ability to separate *Arcobacter* from competitive flora (Kulkarni et al., 2002; Ongor et al., 2004; Merga et al., 2011). In terms of *Arcobacter* identification, multiplex polymerase chain reaction is the most common

method used to identify genus and species of *Arcobacter* (Houf et al., 2000; Gonzalez et al., 2007).

2.6. Genetic characterization of *Arcobacter*

Many molecular techniques such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), repetitive sequence-based PCR (rep-PCR), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) have been used to identify subtypes and genetic relatedness of *Arcobacter* strains (Houf et al., 2002a; On et al., 2004; Ho et al., 2008; Ferreira et al., 2013; Alonso et al., 2014). Among available molecular techniques, rep-PCR has shown to be suitable for *Arcobacter* genotyping. This method had high discriminatory power and reproducibility (Phasipol et al., 2013). Rep-PCR was not only fast, low cost, easy to perform and interpret, and suitable for characterization of large numbers of *Arcobacter* isolates, but it also provided acceptable results that can help differentiate closely related strains of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Houf et al., 2002a).

2.7. *Arcobacter* in Thailand

The information of *Arcobacter* prevalence and infection in Thailand is limited. A few studies reported a high prevalence of *Arcobacter* in ground chicken meat samples collected from retail markets (Atabay et al., 2003; Vindigni et al., 2007). *Arcobacter* was isolated from meat samples more frequently than *Campylobacter* (Vindigni et al., 2007; Bodhidatta et al., 2013). The illness associated with consumption of food contaminated with *Arcobacter* at the restaurant was 13% per meal eaten and increased to 75% when ten meals were consumed. In addition, *Arcobacter* could be isolated from stool samples of diarrheic patients (Taylor et al., 1991). This organism was also found in environmental samples (e.g. river water and

canal water) in Japan and Thailand (Morita et al., 2004). Recently, there were some documents regarding the prevalence of *Arcobacter* in Thailand, but the information on the occurrence of *Arcobacter* in poultry processing plants has not yet been investigated.

CHAPTER III
MATERIALS AND METHODS

3.1. Sample collection

In this study, a total of 388 environmental and chicken-related samples were collected from 2 poultry processing plants (A and B). Plant A is located in Chachoengsao province with a capacity of less than 10,000 birds per day. This plant processes in one shift which starts at 6 a.m. and works 8 hours a day or until the last flock is slaughtered. Plant A is cleaned and sanitized after the shift ends. Plant B is located in Samutsakorn province with a capacity of 150,000 birds per day and works in two shifts. The day shift of plant B usually begins at 5 a.m. and ends at 4 p.m., while the night shift starts at 6 p.m. to 3 a.m. with full clean up between shifts. Plant B has been approved for export chicken products to trade partner country. Finished products of plant B are retail meat i.e. boneless breast (BB), bone in leg (BIL), wing, and fillet, while plant A only provides whole chicken carcasses for domestic consumption. Samples from both plants were collected from broiler flocks raised consecutively for two production cycles. On the sampling day, the target flock of plant A was slaughtered in the last and middle batch of the first and the second sampling day, respectively. In contrast, the target flock of plant B was slaughtered as the first batch of the day after the plant was cleaned and sanitized. Samples from both plants were collected before the target flock was slaughtered and during the target flock was slaughtered. Samples from each plant were collected from slaughtering processes starting from hanging, scalding, defeathering, evisceration, I/O washing, chilling to packaging.

For chicken-related samples, samples from cloaca and meat products were collected. Each cloacal sample was taken with a sterile cotton swab and then placed into 10 ml Clary-blair transport medium. Finished products such as chicken wing, fillet, boneless breast (BB), and bone in leg (BIL) were also collected from cutting line

and placed in sterile containers. Chicken carcasses were rinsed with buffered peptone water (BPW) for 1 minute and the rinsate was collected to culture for *Arcobacter*.

For environmental samples, sterile cotton swabs pre-moistened with 10 ml of 0.1% BPW were used to wipe the surface of equipment such as shackles, breast comforters, gloves, evisceration tools, packaging tables, and weights along the processing line and then placed into Cary-Blair transport medium. Additionally, water samples such as tab water, chilling water, and carcass washed water after scalding and evisceration were also taken. Fifty milliliters (50 ml) of each water sample was collected in sterile container. Samples were immediately transported to the laboratory and processed within 4 h after sampling. Sampling scheme of the study is shown in Figure 2.

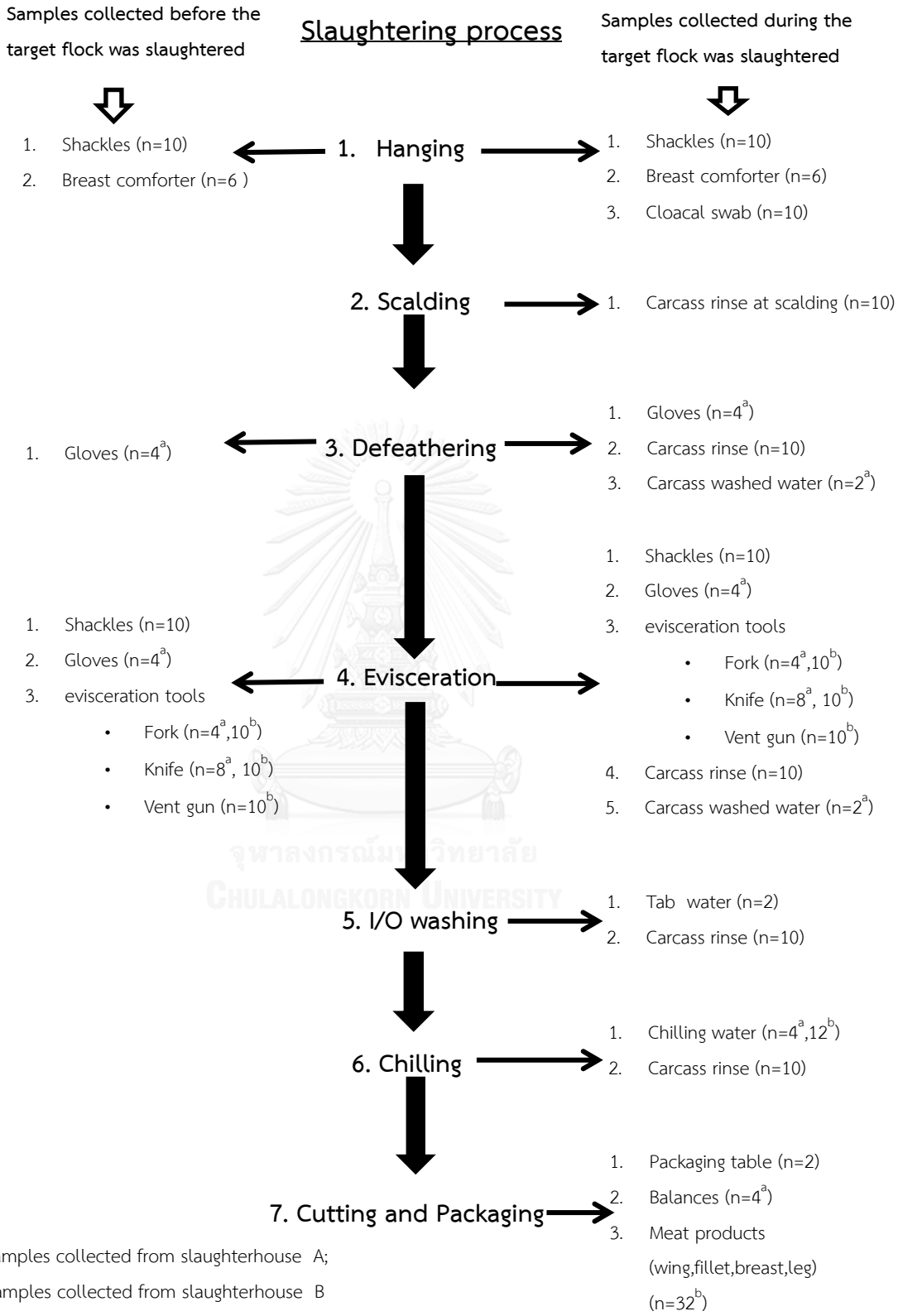


Figure 1. Sample collection scheme

3.2. *Arcobacter* isolation

The isolation of *Arcobacter* spp. was performed by selective enrichment method and membrane filtration method according to the previously published protocol (Atabay et al., 2003) with some modifications. Samples were enriched in *Arcobacter* enrichment broth (AEB) composed of *Arcobacter* enrichment basal medium (Oxoid, CM965; Hampshire, UK) and CAT selective supplement including cefoperazone (8 mg/l), amphotericin (10 mg/l), and teicoplanin (4 mg/l). Twenty milliliters of carcass rinse sample were inoculated into 20 ml of double-strength AEB. Swab samples from cloaca and slaughterhouse environment were transferred into new test tubes containing 10 ml of AEB. Each water sample (20 ml) was added to 20 ml of double-strength AEB (Aydin et al., 2007). Ten grams of meat samples were weighted and suspended in 90 ml of AEB and homogenized in stomacher for 1 min and then approximately 20 ml out of 90 ml of each homogenate were put in sterile container. All samples were incubated at 25°C for 48 hours under aerobic conditions. After enrichment, membrane filtration technique on the modified charcoal cefoperazone deoxycholate agar (mCCDA) was used to seperate *Arcobacter* from competitive microorganisms. Two hundred microliters of enriched samples were inoculated onto a 47 mm diameter 0.45 µm pore size nitrocellulose membrane filter placed on the surface of mCCDA agar plate. The membrane was removed after 30 min. The inoculated agar was incubated at 25°C under aerobic conditions for 48 hours or until the growth of *Arcobacter* colonies was observed (Atabay et al., 2003). Suspected *Arcobacter* colonies (grayish, pin-point colonies) were subcultured onto mCCDA agar plate and incubated for 48 hours at 25 °C under aerobic conditions. Each *Arcobacter* isolate was then identified and preserved at -80 °C in cryovial tube containing skim milk and 30% glycerol.

3.3. *Arcobacter* identification

A multiplex polymerase chain reaction (PCR) was used for *Arcobacter* identification. Briefly, suspected *Arcobacter* colonies were subcultured onto mCCDA agar plate and incubated at 25 °C for 48 hours under aerobic conditions. The

colonies were picked and suspended in 100 µl of sterile distilled water and boiled for 10 min. The suspension was centrifuged for 5 min and supernatant was collected. Multiplex PCR was performed according to the previously published protocol (Douidah et al., 2010). PCR reaction was carried out in a 25-µl reaction mixture composed of 1x PCR buffer (Kapa Biosystems, Boston, USA), 1.5 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphates, 25 pmol of each primer and 0.75U Taq DNA polymerase (Kapa Biosystems, Boston, USA). PCR amplification started with an initial denaturation at 94 °C for 3 min and then 30 cycles of denaturation at 94 °C (45 sec), annealing at 58 °C (45 sec) and extension at 72 °C (2 min), followed by a final extension at 72 °C for 5 min. Primers for *Arcobacter* species-specific multiplex PCR were presented in Table 1. *A. butzleri* NCTC 12481, *A. skirrowii* NCTC 12731, and *A. cryaerophilus* NCTC 11885 were used as positive control strains. PCR products were examined in 1.2% agarose gel. After electrophoresis at 100 V for 30 min, gel was stained with ethidium bromide and visualized in a UV gel document system.

Table 1. Primers for *Arcobacter* species identification (Douidah et al., 2010)

primers		Sequence (5'-3')	Fragment size (bp)
A. Butzleri	ArcoF	GCY AGA GGA AGA GAA ATC AA	2061
	ButR	TCC TGA TAC AAG ATA ATT GTA CG	
A. Skirrowii	ArcoF	GCY AGA GGA AGA GAA ATC AA	198
	SkiR	TCA GGA TAC CAT TAA AGT TAT TGA TG	
A. Cryaerophilus	GyrasF	AGA ACA TCA CTA AAT GAG TTC TCT	395
	GyrasR	CCA ACA ATA TTT CCA GTY TTT GGT	

3.4. Genetic characterization of *Arcobacter*

In this study, rep-PCR with $(GTG)_5$ primers was used to investigate genetic relationship among *Arcobacter* isolates (Chomczynski and Rymaszewski, 2006). Briefly, *Arcobacter* colonies grown on mCCDA agar at 25 °C for 48h under aerobic conditions were suspended into 500 µl of alkaline PEG reagent for cell lysis. Then, the mixture was heated at 90 °C for 10 min and centrifuged at 12,000 rpm for 5 min.

Two microliters of the supernatant were used as DNA template in PCR mixture. The PCR mixture contained 1x PCR buffer, 2 µl of 2.5 mM of each deoxynucletide triphosphates, 20 µM $(GTG)_5$ primers and 0.625 U *Ex Taq* DNA polymerase (Phasipol et al., 2013). PCR conditions consisted of an initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 94 ℃ for 45 sec, annealing at 40 ℃ for 1 min and extension at 65 ℃ for 10 min and a final extension step at 65 ℃ for 20 min. PCR product was verified by gel electrophoresis (1% agarose gel) in 0.5x Tris-borate-EDTA buffer at 120 V for 2.2 hours. Gel was stained with 5 µg/ml ethidium bromide and destained with tap water for 10 min and then visualized by gel scanner (Typhoon 9410, Amersham Pharmacia Biotech Inc., New Jersey, 34 USA). The Gelcompar®II 5.1 software package (Applied Maths, Belgium) was used to determine DNA patterns. Similarity values of the isolates were calculated using Pearson's correlation and constructed by unweighted pair group method using arithmetic mean (UPGMA). The cut-off for clustering was set at 90% similarity.

CHAPTER IV
RESULTS

4.1. Occurrence of *Arcobacter* in two poultry processing plants

A total of 388 environmental and chicken-related samples collected from two poultry processing plants (A and B) were analyzed for the presence of *Arcobacter*. The occurrence of *Arcobacter* in plants A and B was 70.24% and 52.73%, respectively (Table 2). *Arcobacter* was isolated from both environmental and chicken-related samples. *A. butzleri* was the most common *Arcobacter* species (98%) found in this study. No significant difference in the occurrence of *Arcobacter* between 2 processing plants was observed ($p>0.05$). For plant A, approximately 67% and 74% of samples collected from the first and the second sampling days were *Arcobacter* positive, respectively. The prevalence of *Arcobacter* in both environmental and chicken-related samples in two sampling days was shown in Table 3. Among 118 *Arcobacter* positive samples collected from plant A, 115 samples were contaminated with *A. butzleri* and 3 samples were contaminated with *A. skirrowii*. For plant B, the occurrence of *Arcobacter* in the first and the second sampling days was around 53%. The detection rate of *Arcobacter* in plant B was shown in Table 4. All environmental and chicken-related samples in plant B were contaminated with *A. butzleri*, except one sample from carcass rinse at scalding stage that was contaminated with *A. skirrowii*.

To determine the source of *Arcobacter* contamination in poultry slaughterhouses, the slaughtering process was divided into 3 zones as follows: i) dirty zone (live bird, hanging, stunning, killing and bleeding area), ii) medium zone (scalding, defeathering, evisceration and I/O washing area) and iii) clean zone (chilling, cutting and meat product packaging area). The occurrence of *Arcobacter* in both environmental and chicken-related samples in different slaughtering process of

plants A and B was shown in Table 5. For plant A, approximately 38% of samples collected from dirty zone on the first sampling day were *Arcobacter* positive. The contamination rate increased to 75% during processing at medium zone and then reached to 80% at clean zone. Likewise, on the second sampling day, *Arcobacter* contamination rate increased from 52% at dirty zone to 77% at medium zone and to 100% at clean zone. For plant B, approximately 57% of samples collected from dirty zone on the first sampling day were *Arcobacter* positive. Unlike plant A, the contamination rate decreased to 36% at medium zone, but increased to 86% at clean zone. On the second sampling day, *Arcobacter* positive rate was 48% at dirty zone and slightly increased to 49% at medium zone and then reach to 64% at clean zone (Table 5). In general, *Arcobacter* contamination in both slaughterhouses tended to increase throughout multiple slaughtering processes leading to final meat product contamination. Although subsequent I/O washing and chilling stages are commonly used for reducing contaminants on chicken carcasses before cutting and packaging, *Arcobacter* could still be recovered from chicken products.

Table 2. Occurrence of *Arcobacter* in poultry processing plants A and B

Origin of samples	Processing plant A *Arcobacter* positive samples/ No. of samples tested[a]	Processing plant B *Arcobacter* positive samples/ No. of samples tested[a]
Before the target flock was slaughtered		
Breast comforter	3/6	6/6
Shackles at hanging	0/10	6/10
Shackles at evisceration	0/10	0/10
Gloves at defeathering	4/4	N/A
Gloves at evisceration	4/4	N/A
Fork	4/4*	0/10
Knife	0/8	5/10
Vent gun	N/A[b]	1/10
During the target flock was slaughtered		
Chicken-related samples:		
Cloacal swab	3/10	0/10
Carcass rinse at scalding	9/10	9/10*
Carcass rinse at defeathering	10/10	9/10
Carcass rinse at evisceration	10/10	10/10
Carcass rinse at I/O washing	10/10	8/10
Carcass rinse at chilling	10/10	8/10
Meat product from cutting line	N/A	25/32
Environmental samples:		
Breast comforter	6/6	2/6
Shackles at hanging	7/10*	8/10
Shackles at evisceration	10/10	2/10
Gloves at defeathering	3/4	N/A
Gloves at evisceration	4/4	N/A
Fork	4/4	4/10
Knife	4/8	2/10
Vent gun	N/A	2/10
Carcass washed water at defeathering	2/2	N/A
Carcass washed water at evisceration	2/2	N/A
Inside/Outside wash water	1/2	0/2
Chilling water	4/4	8/12
Packaging table	1/2	1/2
Balances	3/4	N/A
Total	**118/168 (70.24%)**	**116/220 (52.73%)**

[a] All *Arcobacter* isolates identified in this study were *A. butzleri,* except for those marked with * were *A. butzleri and A. skirrowii*.

[b] N/A, not applicable.

Table 3. Occurrence of *Arcobacter* in the 1st and 2nd sample collection of poultry processing plant A

Origin of samples	No. of samples collected on each sampling day	No. of *Arcobacter* positive samples[a]	
		1st sampling day	2nd sampling day
Before the target flock was slaughtered			
Breast comforter	3	2	1
Shackles at hanging	5	0	0
Shackles at evisceration	5	0	0
Gloves at defeathering	2	2	2
Gloves at evisceration	2	2	2
Fork	2	2*	2
Knife	4	0	0
During the target flock was slaughtered			
Chicke-related samples:			
Cloacal swab	5	1	2
Carcass rinse at scalding	5	5	4
Carcass rinse at defeathering	5	5	5
Carcass rinse at evisceration	5	5	5
Carcass rinse at I/O washing	5	5	5
Carcass rinse at chilling	5	5	5
Environmental samples:			
Breast comforter	3	3	3
Shackles at hanging	5	2*	5*
Shackles at evisceration	5	5	5
Gloves at defeathering	2	1	2
Gloves at evisceration	2	2	2
Fork	2	2	2
Knife	4	1	3
Carcass washed water after defeathering	1	1	1
Carcass washed water after evisceration	1	1	1
Tab water	1	1	0
Chilling water	2	2	2
Packaging table	1	0	1
Balances	2	1	2
Total	**84**	**56/84 (66.67%)**	**62/84 (73.81%)**

[a] All *Arcobacter* isolates identified in this study were *A. butzleri,* except for those marked with * were *A. butzleri and A. skirrowii*.

Table 4. Occurrence of *Arcobacter* in the 1st and 2nd sample collection of poultry processing plant B

Origin of samples	No. of samples collected on each sampling day	No. of *Arcobacter* positive samples[a]	
		1st sampling day	2nd sampling day
Before the target flock was slaughtered			
Breast comforter	3	3	3
Shackles at hanging	5	3	3
Shackles at evisceration	5	0	0
Fork	5	0	0
Knife	5	0	5
Vent gun	5	1	0
During the target flock was slaughtered			
Chicke-related samples:			
Cloacal swab	5	0	0
Carcass rinse at scalding	5	5	4*
Carcass rinse at defeathering	5	5	4
Carcass rinse at evisceration	5	5	5
Carcass rinse at I/O washing	5	5	3
Carcass rinse at chilling	5	5	3
Meat product from cutting line	16	15	10
Environmental samples:			
Breast comforter	3	2	0
Shackles at hanging	5	4	4
Shackles at evisceration	5	0	2
Fork	5	1	3
Knife	5	0	2
Vent gun	5	0	2
Tab water	1	0	0
Chilling water	6	3	5
Packaging table	1	1	0
Total	**110**	**58/110 (52.73%)**	**58/110 (52.73%)**

[a] All *Arcobacter* isolates identified in this study were *A. butzleri,* except for those marked with * were *A. butzleri and A. skirrowii*.

Table 5. Occurrence of *Arcobacter* in different slaughtering processes of poultry processing plants A and B

Slaughtering stage		Number of positive samples/ No. of examined samples			
		plant A		plant B	
		1st Sampling day	2nd Sampling day	1st Sampling day	2nd Sampling day
(1)	Dirty zone (live bird, hanging, stunning, killing and bleeding area)				
	shackles at hanging	2/10	5/10	7/10	7/10
	breast comforter	5/6	4/6	5/6	3/6
	cloacal swab	1/5	2/5	0/5	0/5
	Total	**8/21 (38.10%)**	**11/21 (52.38%)**	**12/21 (57.14%)**	**10/21 (47.62%)**
(2)	Medium zone (scalding, defeathering, evisceration and I/O washing area)				
	shackles at evisceration	5/10	5/10	0/10	2/10
	gloves at defeathering	3/4	4/4	N/A	N/A
	gloves at evisceration	4/4	4/4	N/A	N/A
	fork	4/4	4/4	1/10	3/10
	knife	1/8	3/8	0/10	7/10
	vent gun	N/A[a]	N/A	1/10	2/10
	carcass washed water at defeathering	1/1	1/1	N/A	N/A
	carcass washed water at evisceration	1/1	1/1	N/A	N/A
	carcass rinse at scalding	5/5	4/5	5/5	4/5
	carcass rinse at defeathering	5/5	5/5	5/5	4/5
	carcass rinse at evisceration	5/5	5/5	5/5	5/5
	carcass rinse at I/O washing	5/5	5/5	5/5	3/5
	tab water	1/1	0/1	0/1	0/1
	Total	**40/53 (75.47%)**	**41/53 (77.36%)**	**22/61 (36.07%)**	**30/61 (49.18%)**
(3)	Clean zone (chilling, cutting and meat product packaging area)				
	chilling water	2/2	2/2	3/6	5/6
	packaging table	0/1	1/1	1/1	0/1
	weights	1/2	2/2	N/A	N/A
	carcass rinse at chilling	5/5	5/5	5/5	3/5
	meat product from cutting line	N/A	N/A	15/16	10/16
	Total	**8/10 (80%)**	**10/10 (100%)**	**24/28 (85.71%)**	**18/28 (64.29%)**

4.2. Genetic profiles of *Arcobacter*

In this study, rep-PCR with GTG_5 primer was performed to determine the genotypes of *Arcobacter* isolated from two poultry processing plants. The similarity between fingerprints was calculated using the Pearson correlation and grouped by using the UPGMA algorithm. The banding patterns obtained were composed of 8 – 15 fragments with the sizes ranging from 300 to 9,000 bp. The phylogenetic analysis of *Arcobacter* banding patterns revealed a wide heterogeneity among isolates (Figure 2). At the similarity level of 90%, 42 and 67 distinct genotypes of *Arcobacter* were found among 118 and 116 *Arcobacter* isolates from plant A and plant B, respectively. The most frequently detected rep-PCR pattern in plant A was A4 pattern comprising of 14 isolates obtained from various sources i.e. carcass rinse from different stages, chilling water, gloves, and knife (Figure 3). However, no dominant rep-PCR pattern was present in plant B.

On the first sampling day of plant A, rep-PCR revealed that 29 genotypes (A1.1-A1.29) were recovered from 56 *Arcobacter* isolates (Figure 4). At the 90% similarity cut off, 7 out of 29 genotypes (A1.1, A1.2, A1.10, A1.14, A1.16, A1.20 and A1.21) contained isolates from both environmental and chicken-related samples and 8 out of 29 genotypes (A1.1, A1.2, A1.5, A1.10, A1.14-15, A1.20 and A1.21) were obtained from various slaughtering stages. The presence of similar *Arcobacter* genotypes in environmental samples and chicken-related samples indicated the possibility of direct contact between carcasses and slaughterhouse environment, which can lead to the spread of *Arcobacter* along the processing line. For example, *Arcobacter* genotype A1.1 was found among carcass rinse at different slaughtering stages starting from scalding to chilling and this genotype was also recovered from environmental samples (i.e. gloves at evisceration step and chilling water). To identify whether *Arcobacter* contamination in the processing plant was originated from chickens, 5 cloacal swab samples were collected. However, only one cloacal swab sample was *Arcobacter* positive. Moreover, *Arcobacter* isolate from this sample had a unique rep-PCR pattern (A1.13) indicating that chicken probably may not be the important source of *Arcobacter* contamination in this processing plant.

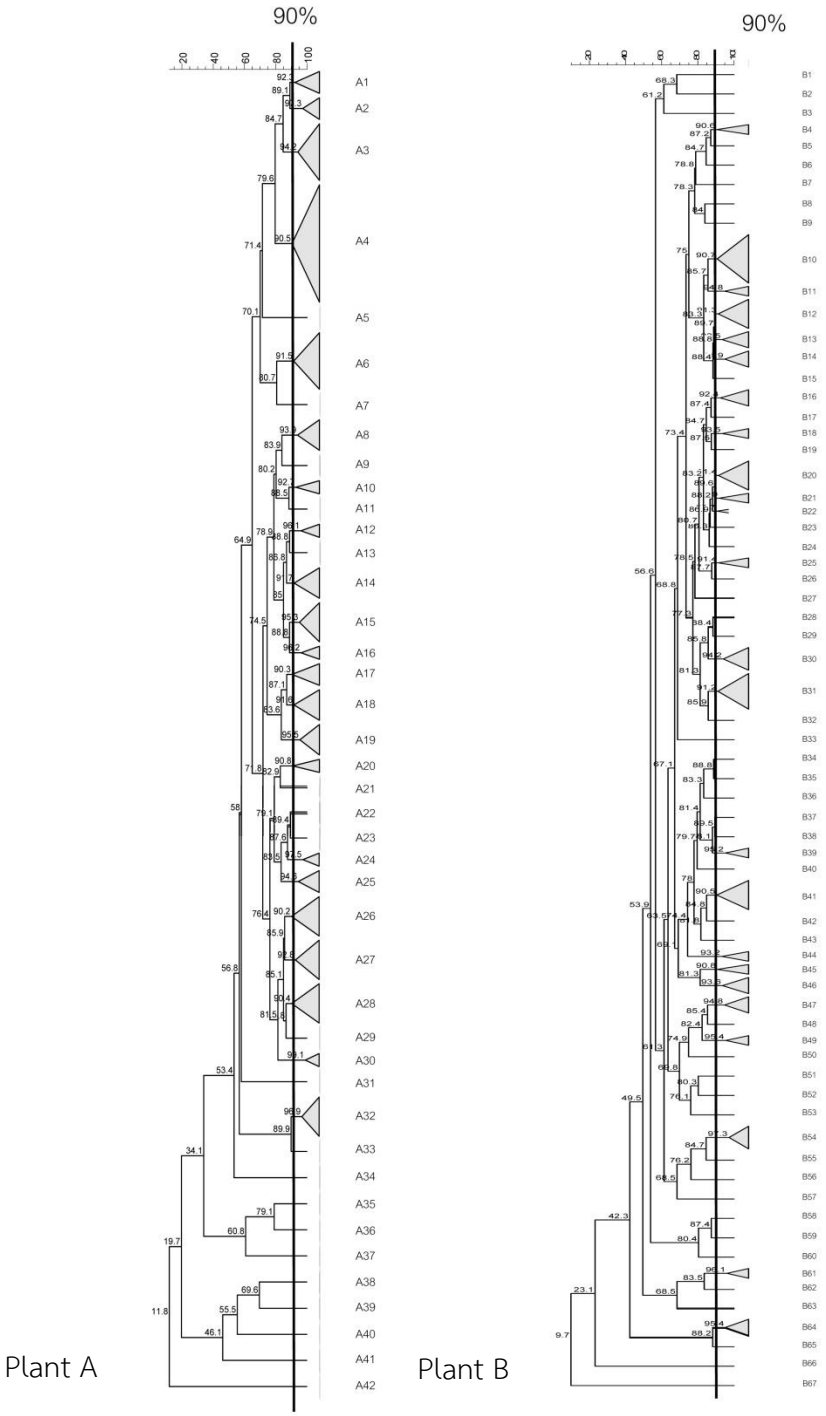
Similar to the first sampling day, *Arcobacter* isolates from the second sampling day of the processing plant A also showed a high genetic diversity (Figure 5). In total, 27 different genotypic patterns (A2.1-A2.27) were obtained. Fifteen out of 27 genotypes (A2.4, A2.6, A2.7, A2.9-11, A2.13-15, A2.21-26) were composed of 2 or more *Arcobacter* isolates. Among these 15 common clusters, 9 clusters (A2.4, A2.7, A2.9-11, A2.14, A2.22, A2.23, and A2.26) were found in both environmental and chicken-related samples suggesting that cross-contamination may occur. Moreover, *Arcobacter* isolates from different slaughtering stages were also clustered into the same genotypes (A2.4, A2.6, A2.7, A2.9-11, A2.14-15, A2.22-24, and A2.26) indicating that *Arcobacter* was widespread in the slaughterhouse environment along the processing line and may lead to chicken products contamination. In terms of *Arcobacter* isolation from cloacal swab samples, 2 genotypes were observed. One isolate was clustered into the same genotype with the isolate from environmental sample at the packaging stage (A2.7), whereas the other isolate had distinct genotype (A2.2). Since no related genotypes between cloacal isolates and chicken products were observed, the intestinal tract should not be considered as the main source of *Arcobacter* contamination in finished products.

On the first sampling day of plant B, 58 *Arcobacter* strains were divided into 35 distinct genotypes (B1.1-B1.35) (Figure 6). Among these 35 genotypes, 23 unique and 12 common rep-PCR patterns were identified. From the 12 common genotypes (B1.1, B1.3, B1.5, B1.9, B1.12-13, B1.15-16, B1.19, B1.23, B1.26, and B1.34), 5 genotypes (B1.3, B1.5, B1.9, B1.23, and B1.34) were observed in both environmental and chicken-related samples. In addition, certain rep-PCR patterns (B1.3, B1.5, B1.9, B1.13, and B1.19, B1.23, and B1.34) were found among *Arcobacter* isolates from different slaughtering stages. These findings indicate that cross-contamination between environment and chicken carcasses may occur during processing along the slaughtering line.

On the second sampling day of plant B, the cluster analysis revealed that 58 *Arcobacter* isolates were grouped into 37 patterns (B2.1-B2.37) (Figure 7). Six clusters (B2.9, B2.17, B2.20, B2.22, B2.32, and B2.33) were composed of *Arcobacter* isolates

from both environmental and chicken-related samples. Moreover, certain patterns (B2.7, B2.9, B2.15, B2.17, B2.20, B2.22, B2.32, B2.33, and B2.36) were recovered from different slaughtering stages. For example, genotype B2.17 was consisted of *Arcobacter* isolates from isolates of carcass rinse from different stages (i.e. defeathering, evisceration, I/O washing), finished product (BB) and processing plant environment (i.e. shackles at hanging and evisceration steps). Our study demonstrated that cross-contamination between slaughterhouse environment and chicken-related samples may occur during poultry processing.

In addition, 14 rep-PCR patterns obtained from the first sampling day of plant A and 9 rep-PCR patterns obtained from the first sampling day of plant B were also recovered on the second sampling day of plants A and B, respectively (Figures 8 and 9). These findings demonstrated that certain *Arcobacter* strains could exist and circulate in the slaughterhouse environment.



*The vertical line indicates the delineation level of 90%.

Figure 2. Cluster analysis of *Arcobacter* isolated from poultry processing plants A and B.

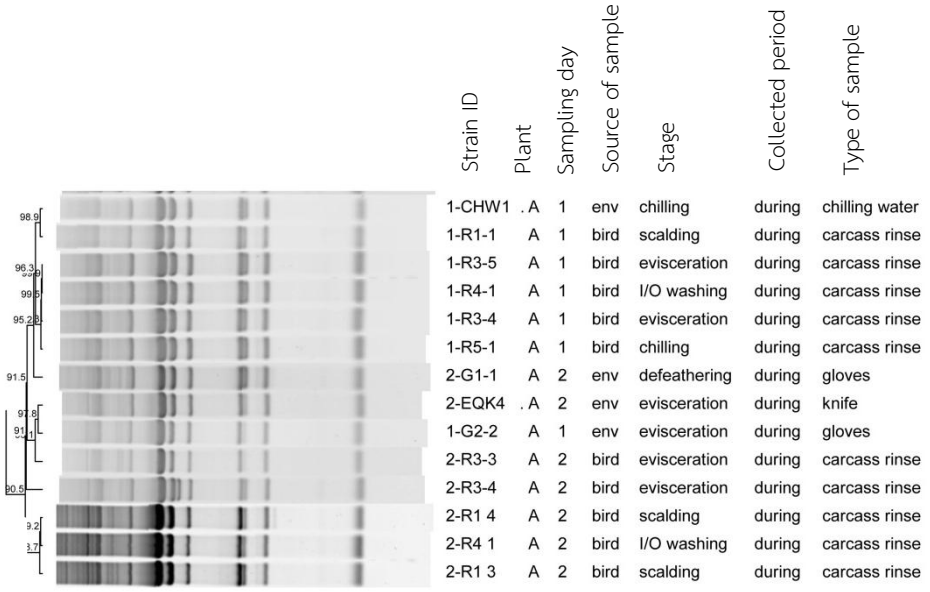


Figure 3. Cluster analysis of *Arcobacter* A4 pattern obtained from poultry processing plant A. The first column shows sample ID. The second column shows poultry processing plant where samples were collected. The third column shows the period of sample collection (1 = 1st sampling day, 2 = 2nd sampling day). The fourth column shows the source of samples (env = environmental samples, bird = chicken-related samples). The fifth column shows the stage of slaughtering process. The last two columns show sample collected period (before the target flock was slaughtered or during the target flock was slaughtered) and the type of sample, respectively.

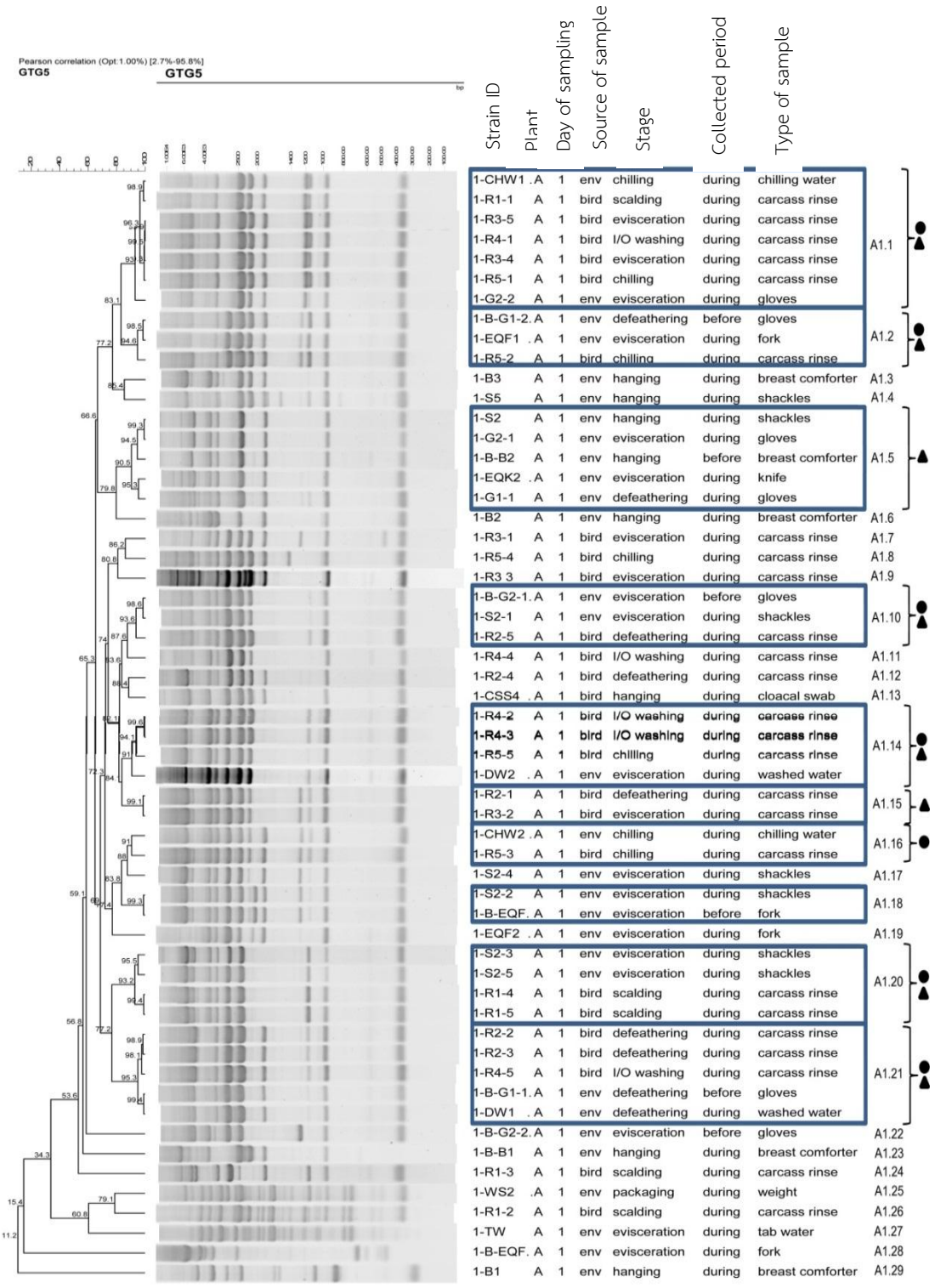


Figure 4. Dendrogram of *Arcobacter* isolates from poultry processing plant A (the first sampling day). The box represents the cluster with 90% similarity cut-off. Genotypes obtained were labeled A1.1-A1.29. (●) The isolates were recovered from both environmental and chicken-related samples. (▲) The isolates were recovered from different slaughtering stages.

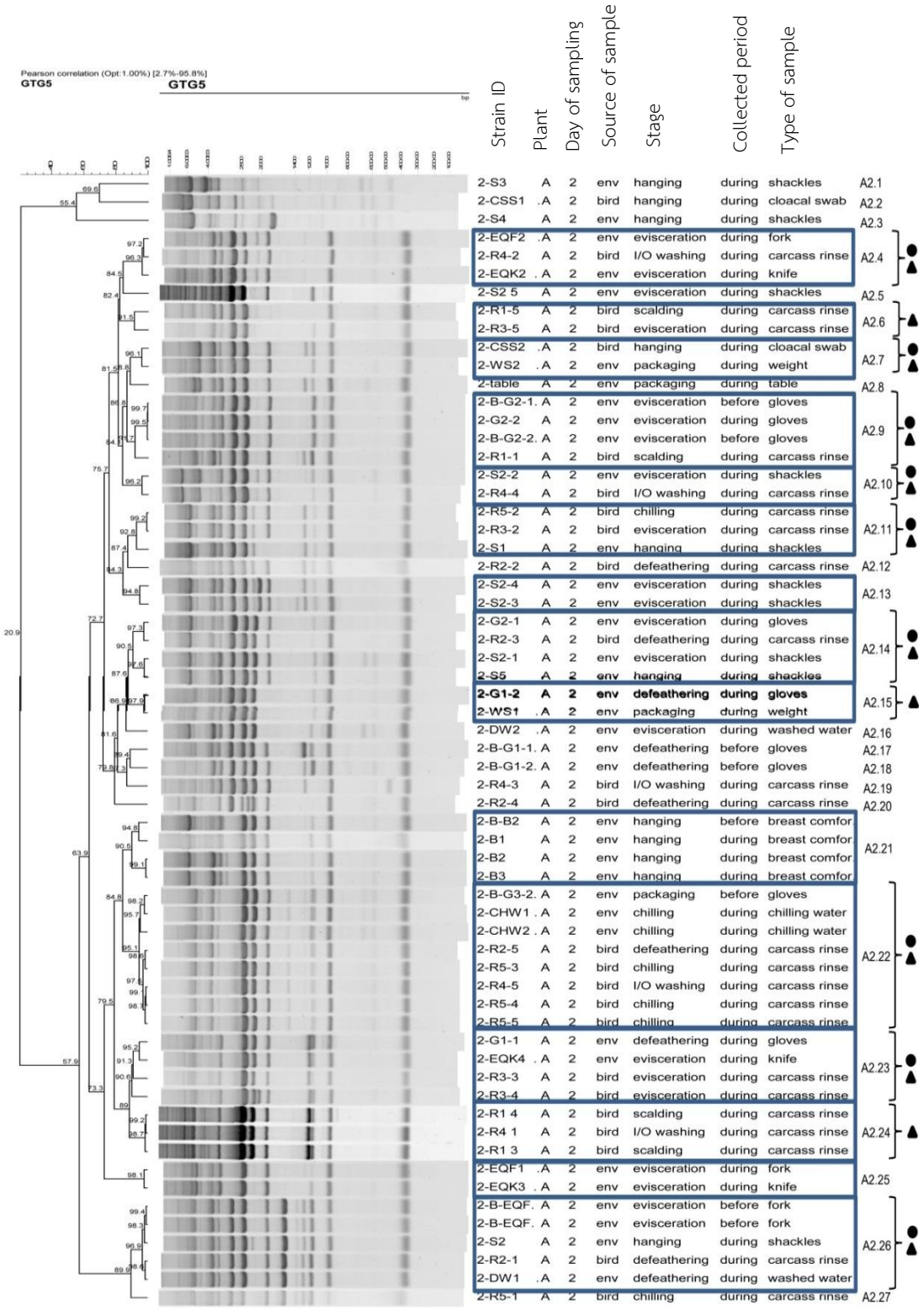


Figure 5. Dendrogram of *Arcobacter* isolates from poultry processing plant A (the second sampling day). The box represents the cluster with 90% similarity cut-off. Genotypes obtained were labeled A2.1-A2.27. (●) The isolates were recovered from both environmental and chicken-related samples. (▲) The isolates were recovered from different slaughtering stages.

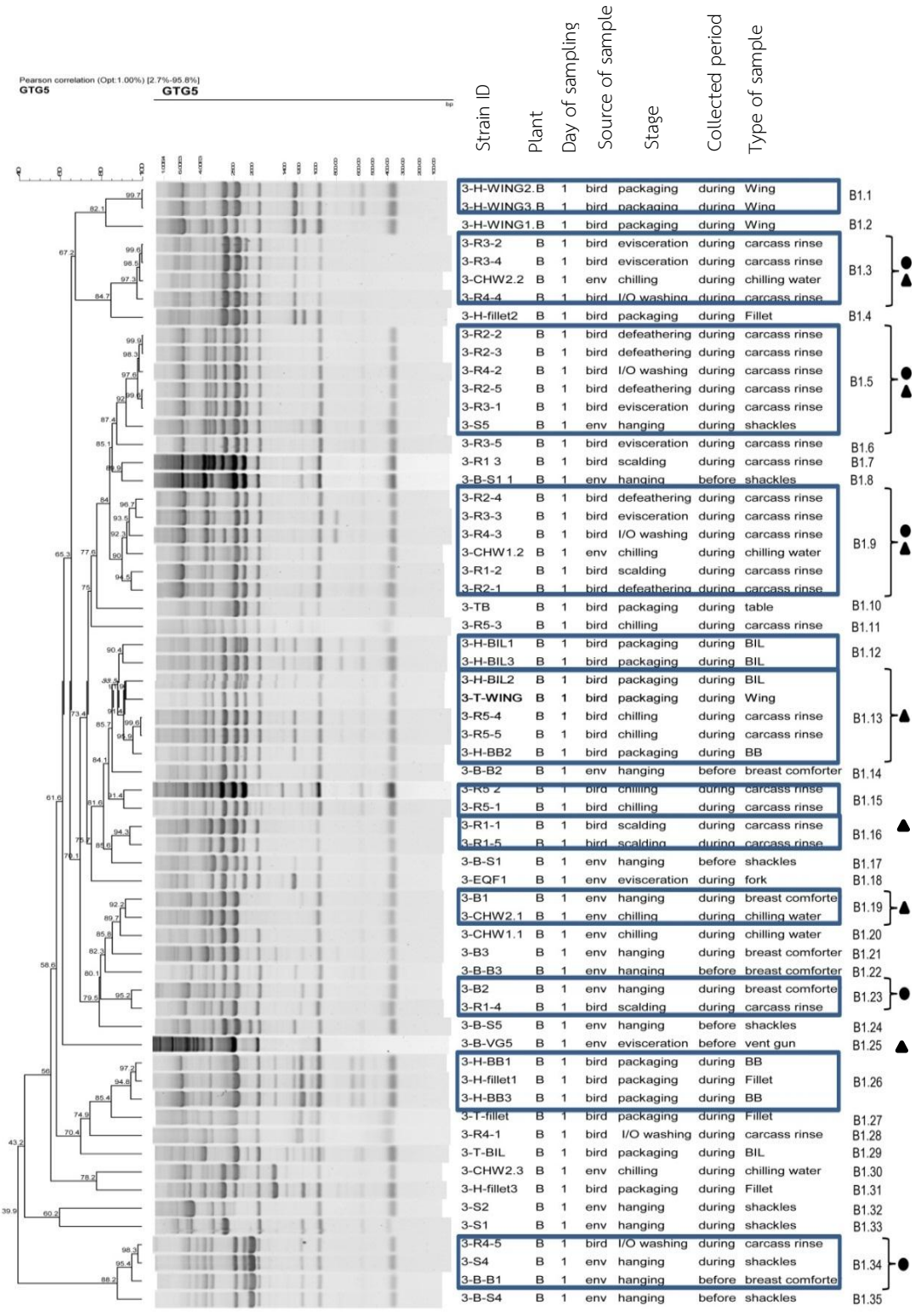


Figure 6. Dendrogram of *Arcobacter* isolates from poultry processing plant B (the first sampling day). The box represents the cluster with 90% similarity cut-off. Genotypes obtained are labeled B1.1-B1.35. (●) The isolates were recovered from both environmental and chicken-related samples. (▲) The isolates were recovered from different slaughtering stages.

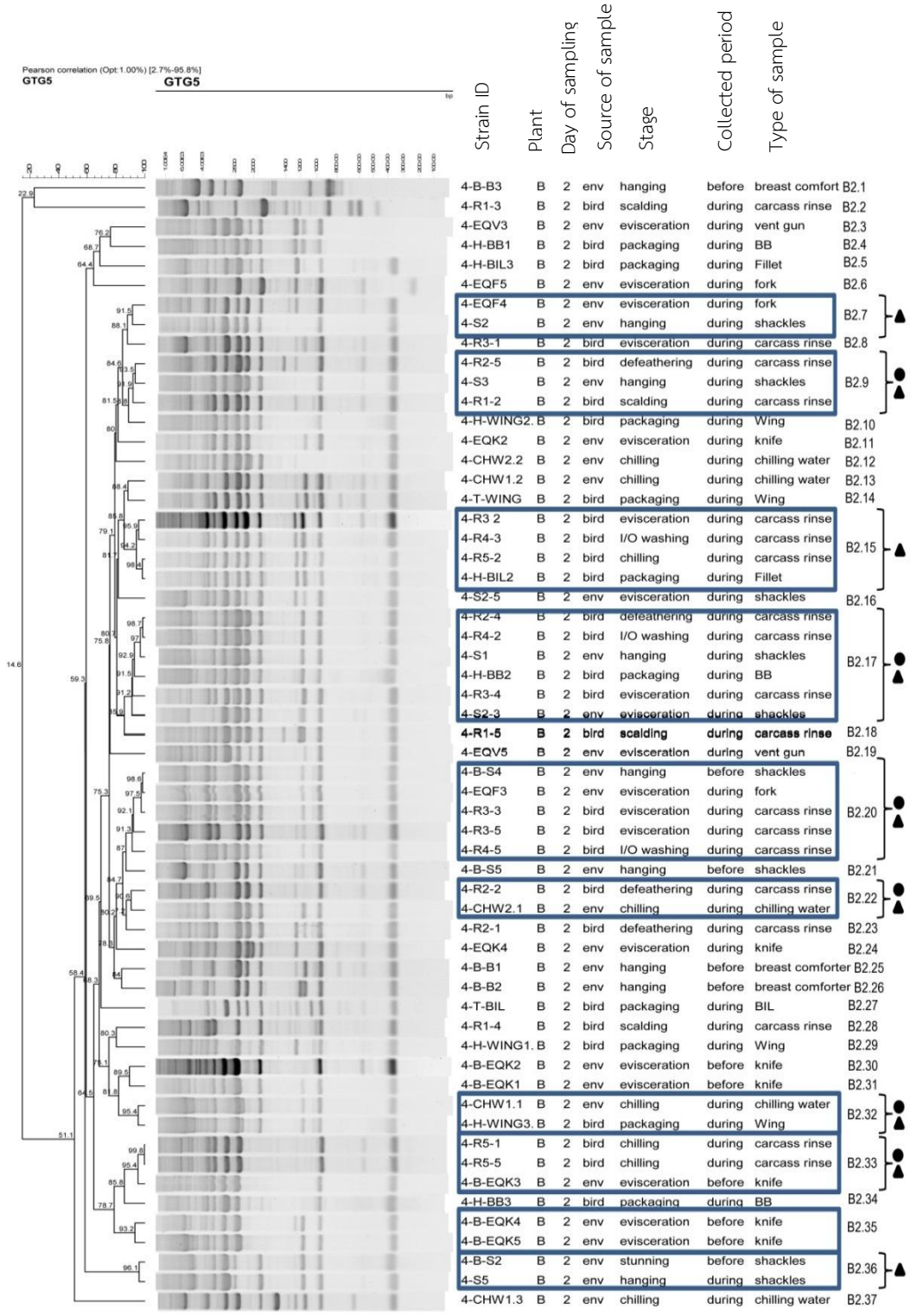


Figure 7. Dendrogram of *Arcobacter* isolates from poultry processing plant B (the second sampling day). The box represents the cluster with 90% similarity cut-off. Genotypes obtained are labeled B2.1-B2.37. (●) The isolates were recovered from both environmental and chicken-related samples. (▲) The isolates were recovered from different slaughtering stages.

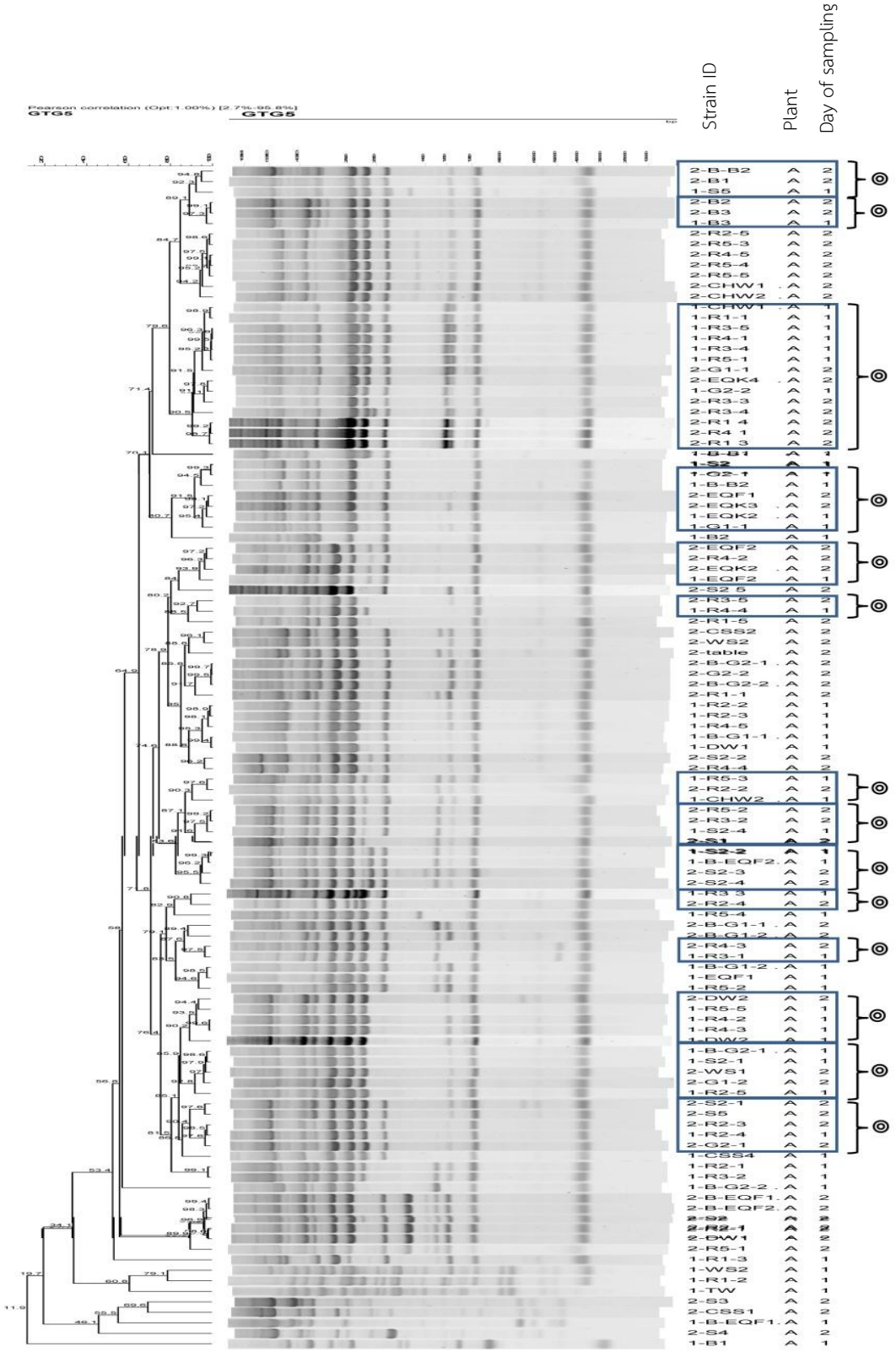


Figure 8. Rep-PCR profiles of *Arcobacter* isolates from the poultry processing plant A. The box represents the cluster with 90% similarity cut-off. (◎) The isolates from the first and the second sampling days were clustered into the same rep-PCR patterns.

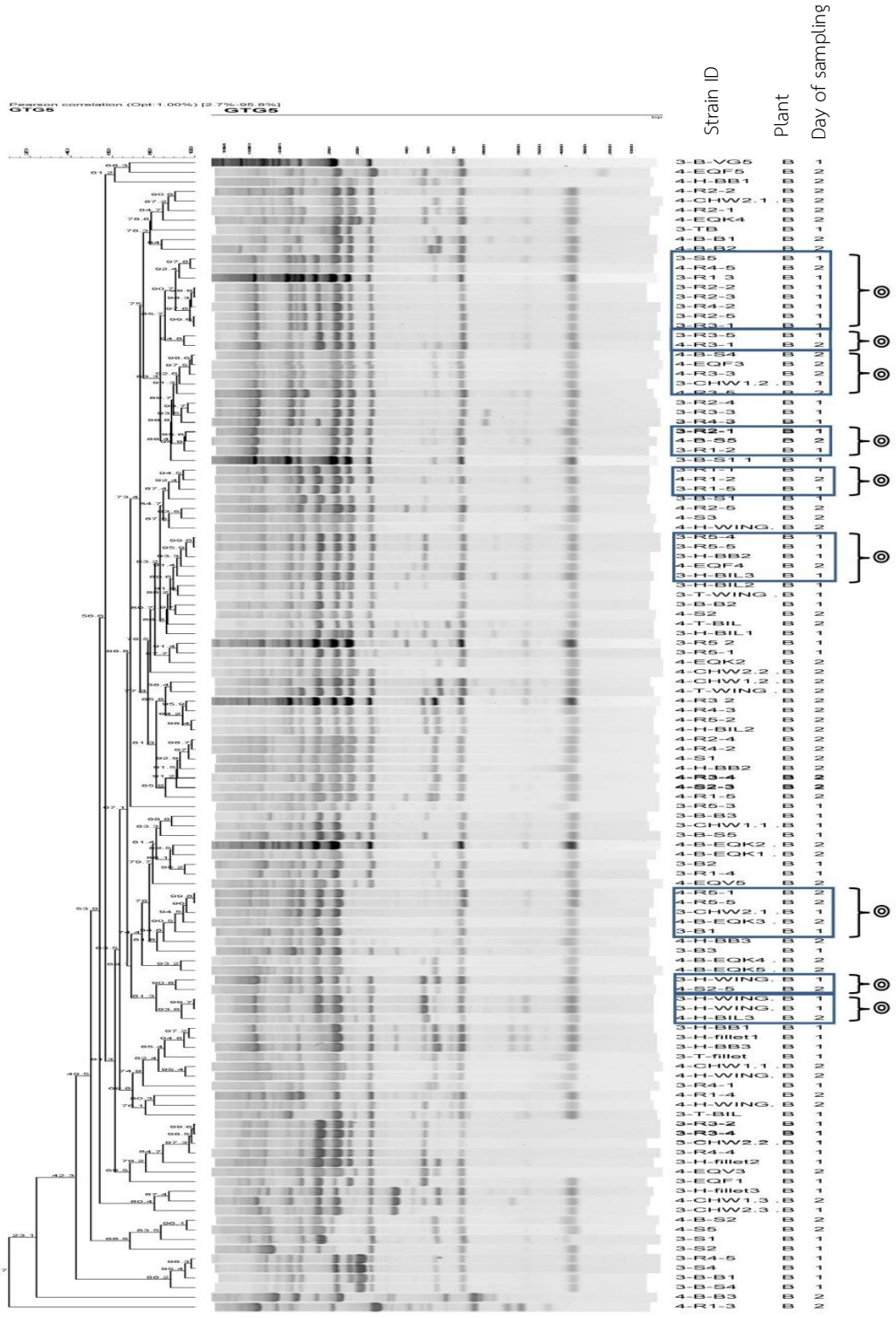


Figure 9. Rep-PCR profiles of *Arcobacter* isolates from the poultry processing plant B. The box represents the cluster with 90% similarity cut-off. (◎) The isolates from the first and the second sampling days were clustered into the same rep-PCR patterns.

4.3. Potential source of *Arcobacter* contamination in chicken products

To investigate the potential source of *Arcobacter* contamination in chicken products, finished products from two poultry processing plants were collected and traced back for the source of *Arcobacter* contamination during slaughtering processes. Plant A currently sells only whole chicken carcasses, whereas chickens from plant B are portioned into retail products. Ten whole carcass rinses after chilling were collected from plant A. For plant B, 25 samples of meat products along the cutting line (i.e. boneless breast (BB), bone in leg (BIL), wing, and fillet) were collected.

In plant A, 5 *Arcobacter* isolates from finished products on the first sampling day were clustered into 5 different patterns. Four patterns were also found in the slaughterhouse environment and carcass rinse at previous stages (pattern A1.1, A1.2, A1.14, and A1.16 in Figure 10). Only one isolate produced a distinct genotype (A1.8). These findings suggested that *Arcobacter* strains from previous slaughtering stages could be transferred to finished products via direct contact with contaminated surface along the processing line. On the second sampling day, 5 *Arcobacter* isolates from finished products were characterized into 3 different patterns. One isolate had a unique banding pattern, while the other 4 isolates were clustered into 2 patterns (A2.11 and A2.22). Pattern A2.11 was seen in finished product as well as in carcass rinse at evisceration step and processing plant environment (i.e. shackles at hanging). For pattern A2.22, 3 isolates from finished products were clustered into the same genotype with the isolates from gloves, chilling water, and carcass rinse at defeathering step (Figure 11). These results indicated that chicken carcasses may be contaminated with *Arcobacter* along the processing line.

For plant B, 10 *Arcobacter* isolates from finished products on the first sampling day were clustered into 4 different patterns (B1.1, B1.12, B1.13, and B1.26), whereas 5 isolates yielded individual patterns. These 4 common genotypes (Figure 12) were found only among *Arcobacter* isolates from chicken-related samples. None of these genotypes were noticed in environmental isolates from previous slaughtering stages. Genotypes B1.1 and B1.12 were found only in wing and BIL

isolates, respectively, while genotypes B1.13 and B1.26 were composed of isolates from various finished products (i.e. BB, fillet, BIL, and wing). In addition to finished products, genotype B1.13 was also noticed among isolates from carcass rinse at chilling stage. These results suggested that chilling and cut-up area could be the point where *Arcobacter* contamination in finished products took place. Among 10 *Arcobacter* isolates from finished products on the second sampling day of plant B, only 3 isolates were clustered into the same genotypes with the isolates from previous slaughtering stages (B2.15, B2.17, and B2.32). The other 7 isolates had 7 unique rep-PCR patterns. Genotype B2.15 was detected among the isolates from finished products and carcass rinse from previous stages, whereas genotype B2.17 was found among the isolates from finished products, carcass rinses, and environmental samples such as shackles at the hanging stage and genotype B2.32 was found among the isolates from finished product and chilling water (Figure 13). These findings suggested that chicken samples might become contaminated with *Arcobacter* by direct contact with slaughterhouse environment and then cross contaminated to other carcasses after they were submerged in the chilling tank.

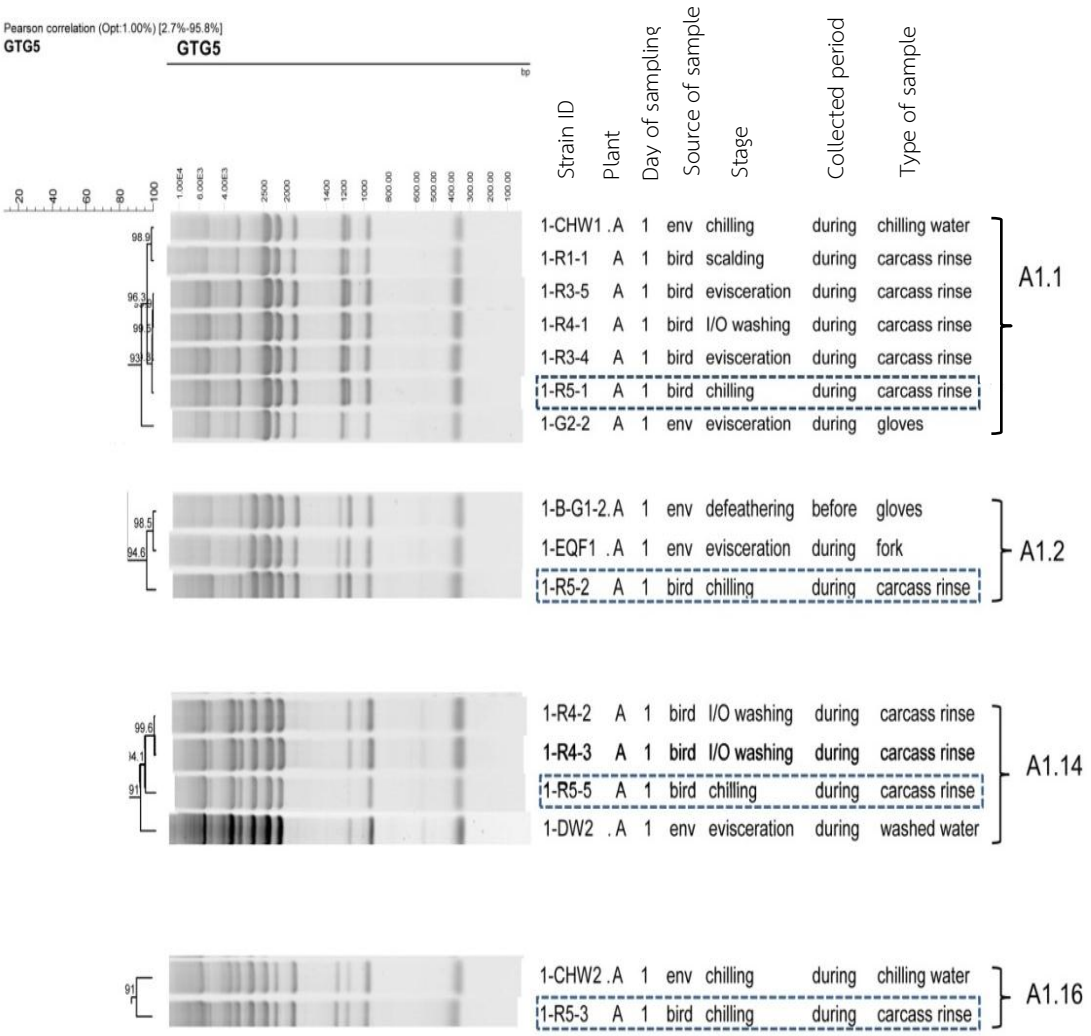


Figure 10. Cluster analysis of *Arcobacter* strains isolated from finished products on the first sampling day of plant A. The dotted-box represents the isolate from finished products.

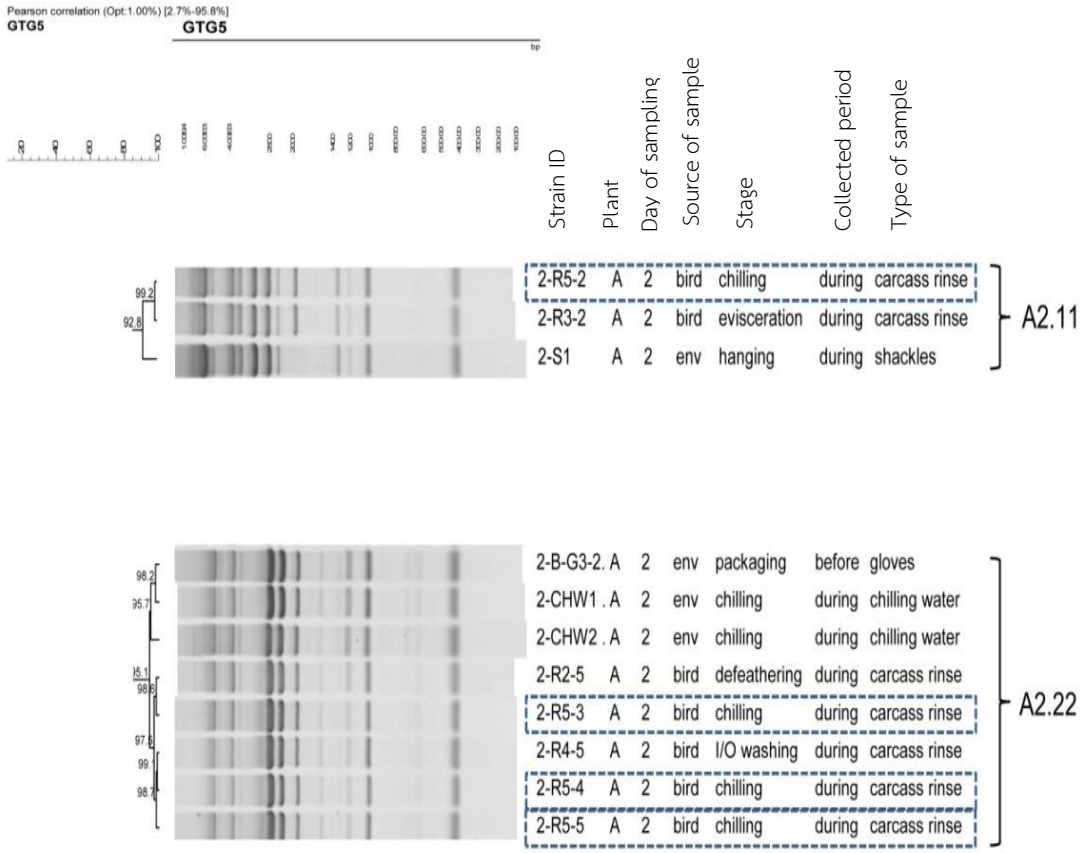


Figure 11. Cluster analysis of *Arcobacter* strains isolated from finished products on the second sampling day of plant A. The dotted-box represents the isolate from finished products.

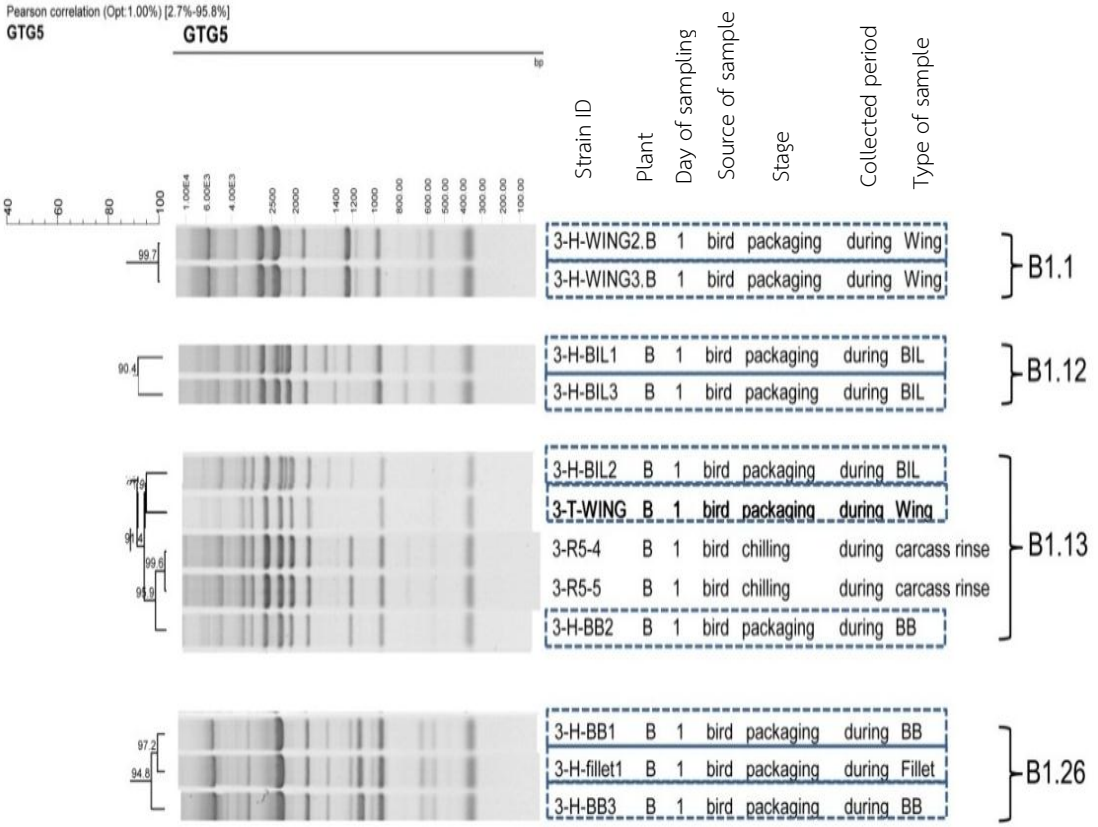


Figure 12. Cluster analysis of *Arcobacter* strains isolated from finished products on the first sampling day of plant B. The dotted-box represents the isolate from finished products.

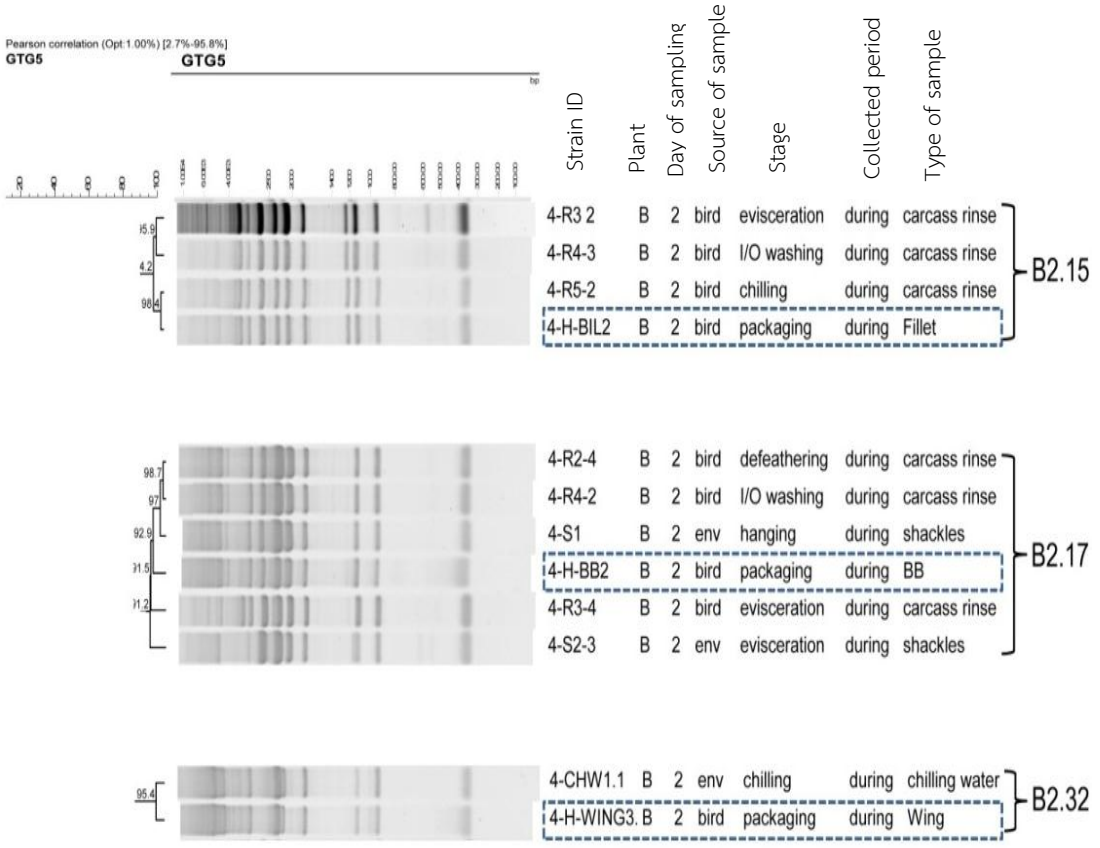


Figure 13. Cluster analysis of *Arcobacter* strains isolated from finished products on the second sampling day of plant B. The dotted-box represents the isolate from finished products.

In plant A, the sampled flocks were slaughtered as the last batch of the first sampling day and the third batch of the second sampling day with no cleaning between batches, while the sampled flocks in plant B were slaughtered as the first batch on both sampling days. Environmental samples (breast comforter, shackles, gloves, and evisceration tools) before the target flock was slaughtered in both plants were sampled to evaluate the efficiency of cleaning and disinfection and to determine the possibility of *Arcobacter* cross-contamination during processing. On the first sampling day of plant A, *Arcobacter* isolates from carcass rinse from various slaughtering stages had the same genotypes with *Arcobacter* isolates from processing plant environment before the target flock was slaughtered (see pattern A1.2, A1.10, and A1.21 in Figure 14). The slaughterhouse environment was likely contaminated with *Arcobacter* from previous positive flocks as there was no cleaning between batches. On the second sampling day, carcass rinse and environmental samples collected before the target flock was slaughtered were clustered into 3 different patterns (A2.9, A2.22, and A2.26) (Figure 15). For example, the isolate from gloves collected before the target flock was slaughtered was clustered into the same rep-PCR pattern (A2.9) with the isolate from carcass rinse at the scalding step. This finding suggested that cross contamination between the slaughterhouse environment and chicken-related sample was occurred.

On the first sampling day of plant B, the cross contamination between the slaughterhouse environment (breast comforter) before the target flock was slaughtered and chicken-related sample (carcass rinse at I/O washing step) was also observed (see pattern B1.34 in Figure 16). On the second sampling day, the isolates obtained from carcass rinse at evisceration, I/O washing, and chilling stages were clustered into the same genotype with the isolates from shackles and knife collected before the target flock was slaughtered (pattern B2.20 and B2.23 in Figure 17). Although the target flocks of plant B were slaughtered as the first batch after the slaughtering line was fully cleaned up, chicken carcasses were still contaminated with *Arcobacter*. These results demonstrated that cleaning and disinfection program

used in the poultry processing plant B might not be effective enough to completely eliminate *Arcobacter*.

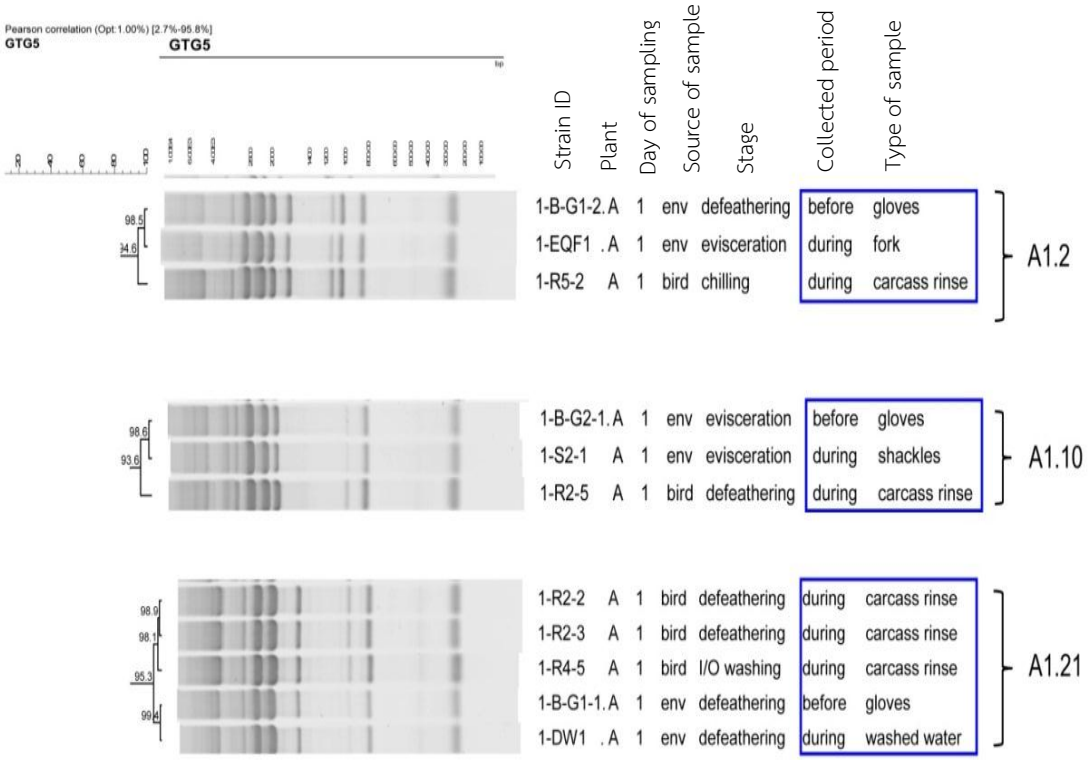


Figure 14. Cluster analysis of *Arcobacter* strains isolated from environmental samples before the target flock was slaughtered and *Arcobacter* strains isolated from the target flock during slaughtering on the first sampling day of plant A. The box represents the cluster with 90% similarity cut-off.

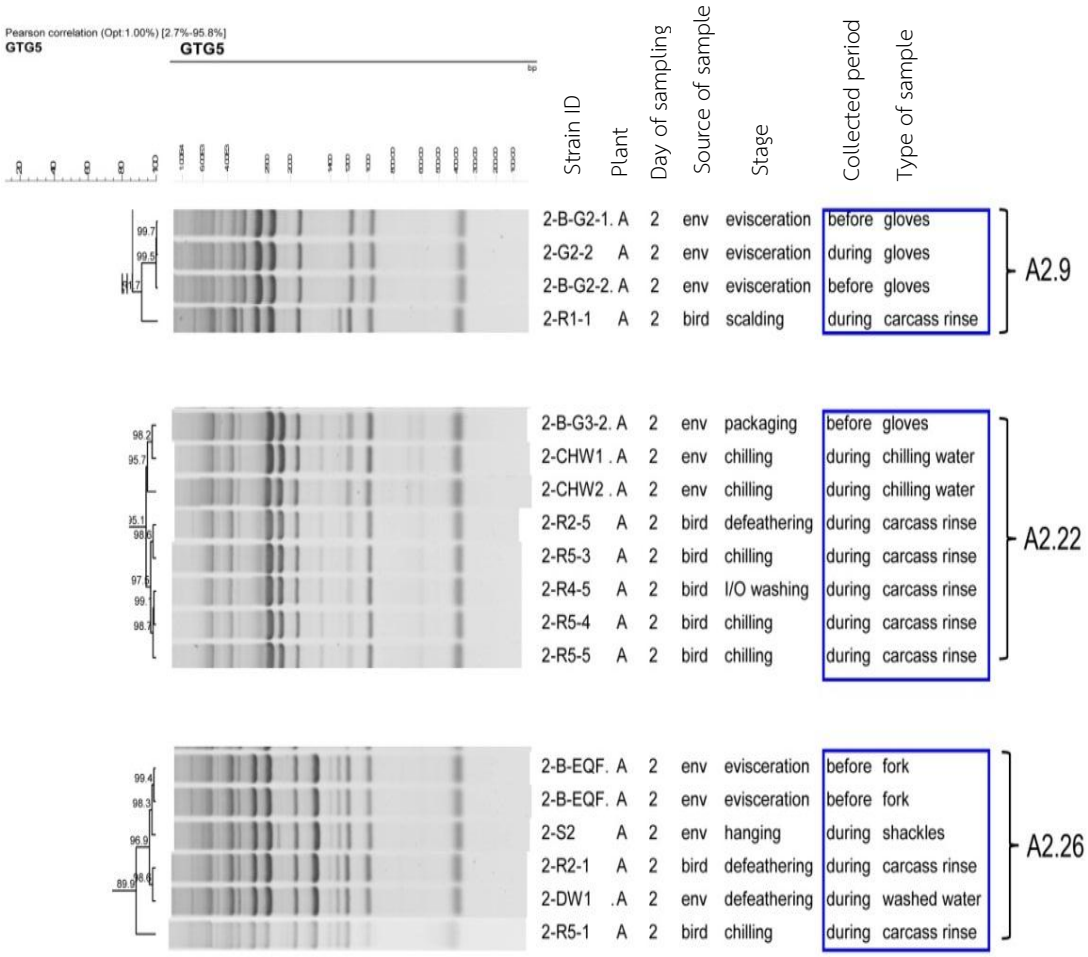


Figure 15. Cluster analysis of *Arcobacter* strains isolated from environmental samples before the target flock was slaughtered and *Arcobacter* strains isolated from the target flock during slaughtering on the second sampling day of plant A. The box represents the cluster with 90% similarity cut-off.

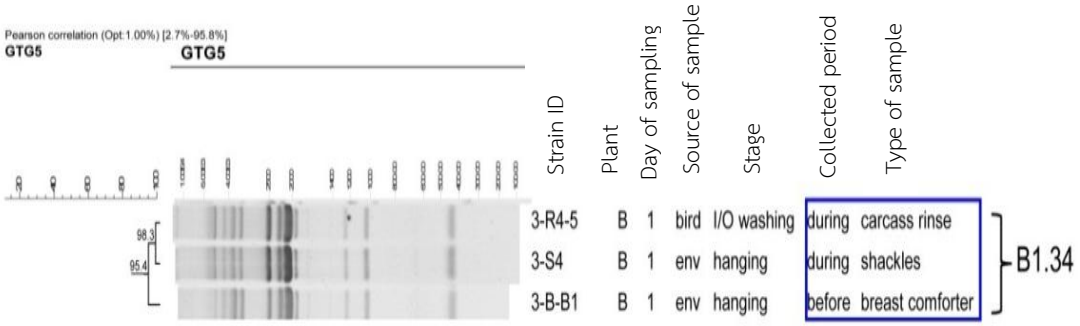


Figure 16. Cluster analysis of *Arcobacter* strains isolated from environmental samples before the target flock was slaughtered and *Arcobacter* strains isolated from the target flock during slaughtering on the first sampling day of plant B. The box represents the cluster with 90% similarity cut-off.

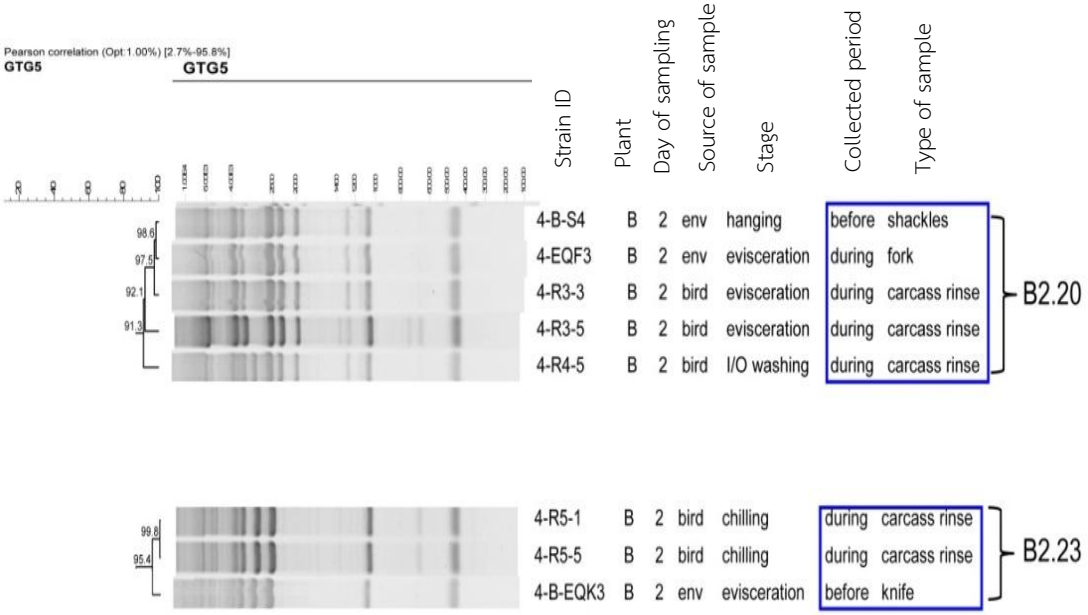


Figure 17. Cluster analysis of *Arcobacter* strains isolated from environmental samples before the target flock was slaughtered and *Arcobacter* strains isolated from the target flock during slaughtering on the second sampling day of plant B. The box represents the cluster with 90% similarity cut-off.

CHAPTER V
DISCUSSION

This study provides information on the occurrence, genetic profiles and potential sources of *Arcobacter* contamination in chicken meat from two poultry processing plants in Thailand. The occurrence of *Arcobacter* in processing plant A was approximately 67% and 74% on the first and the second sampling days, respectively, while the occurrence of this organism in plant B was 53% on both sampling days. The occurrence of *Arcobacter* in poultry processing plants was previously reported in other countries such as Belgium (85%), Turkey (43%), and Iran (45%) (Houf et al., 2002b; Atanassova et al., 2008; Khoshbakht et al., 2014).

In agreement with previous reports (Houf et al., 2002b; Atanassova et al., 2008), *Arcobacter butzleri* is the most common species in poultry processing plants participating in this study. Only few *Arcobacter* isolates in the present study were identified as *A. skirrowii*, whereas no *A. cryaerophillus* was detected. Because *A. butzleri* grew faster than *A. skirrowii* and *A. cryaerophillus* under aerobic conditions and was more resistant to antimicrobials used in isolation media (Atabay et al., 2002; Arias et al., 2011), this may be the explanation why *A. butzleri* was more frequently isolated from environmental and chicken-related samples in this study than other *Arcobacter* species.

The occurrence of *Arcobacter* in both environmental samples and chicken-related samples gradually increased during slaughtering processes from unclean to clean area in both plants. Slaughterhouse environment may become contaminated with *Arcobacter* and contribute to the spread of this microorganism to chicken carcasses. Previous study suggested that the occurrence of *Arcobacter* in chicken carcasses could be detected very early during processing and the contamination tended to increase after passing through various slaughtering stages (Gude et al.,

2005). Another study reported that the prevalence of *Arcobacter* on broiler carcasses increased after chilling (Atanassova et al., 2008). *Arcobacter* can form biofilm to enhance their survival under chill conditions. The presence of biofilms in slaughtering equipment indicated a potential problem because it can help protect *Arcobacter* from being eliminated during cleaning and disinfection. To minimize this problem, the equipment should be cleaned more frequently to remove all organic materials that could be the origin of biofilm formation.

Differences in the occurrence of *Arcobacter* between plant A and plant B could be due to different slaughtering practices. Plant A is a small-scale slaughterhouse that provides whole chicken carcasses for domestic markets, while plant B is a large-scale poultry processing plant that produces many retail products for export. Slaughtering procedure in plant A is generally performed by human, whereas plant B uses modern slaughtering machine and equipment. Although the occurrence of *Arcobacter* in plant A was higher than plant B, no significant difference in *Arcobacter* contamination rates was observed between these plants.

Rep-PCR with GTG_5 primers has been shown to be a useful technique for genotyping the related strains of *Arcobacter* spp. (Collado and Figueras, 2011). At the 90% similarity cut-off, 29 and 27 genotypes were detected from *Arcobacter* isolated from samples collected from the first and the second sampling days of plant A, respectively. In plant B, *Arcobacter* positive isolates from each sampling day yielded 35 and 37 genotypes for the first and the second sampling days, respectively. The presence of high genetic diversity might indicate that there were multiple sources of *Arcobacter* contamination in poultry processing plants (Houf et al., 2003; Son et al., 2006; Aydin et al., 2007; Van Driessche and Houf, 2007). The close contact between slaughterhouse environment and chicken carcasses during processing is probably the transmission route of *Arcobacter* to chicken products (Van Driessche and Houf, 2007). In this study, samples from both plants were collected twice from two sampling days which were several weeks apart. The results revealed that some of the samples collected from the first and the second sampling days were contaminated with the same genotypes. Because *Arcobacter* can adhere to surface (Assanta et al., 2002) and

form biofilm (Kjeldgaard et al., 2009; Ferreira et al., 2013), it may be able to survive in the slaughterhouse environment and distribute to different sites of processing plants even after cleaning and disinfection procedures were performed which lead to carcass contamination and persistence of this organism in the slaughterhouse environment (Houf et al., 2002b).

Characterization of *Arcobacter* strains present in chicken products would help trace the potential sources of contamination. In this study, chicken-related samples from carcass rinse and finished products were contaminated with *Arcobacter*, while the presence of this microorganism in cloacal swab samples was rare. In this study, cloacal swab was collected to provide the evidence of *Arcobacter* colonization in chicken intestinal tract. Only 3 out of 10 cloacal swab samples collected from plant A were *Arcobacter* positive, whereas no *Arcobacter* was found in cloacal swab samples collected from plant B. In addition, rep-PCR patterns of these 3 *Arcobacter* isolates from cloacal swab samples were different from those of chicken-related samples. These findings suggested that chicken intestinal tract should not be the main source of *Arcobacter* contamination in slaughterhouse. Likewise, water used in poultry processing plants should not be the source of *Arcobacter* contamination in chicken carcasses because only 1 out of 4 water samples in this study was *Arcobacter* positive. Moreover, this particular isolate also had a unique genotype, which was different from any other collected samples including chicken products.

The presence of *Arcobacter* in environmental samples before the target flock was slaughtered can lead to chicken carcass contamination during processing. Our results showed that *Arcobacter* genotypes obtained from chicken products were similar to the genotypes found in environmental samples before the target flock was slaughtered indicating that the slaughterhouse environment was a vehicle for cross contamination during processing. Furthermore, *Arcobacter* strains may remain in the slaughterhouse environment due to improper cleaning and disinfection between batches. The importance of surface contact in spreading this foodborne pathogen to finished products should be concerned. Proper cleaning and sanitizing procedures in poultry processing plants must be performed in order to reduce *Arcobacter*

contamination in chicken products during processing to ensure the safety of chicken meat for consumption (Houf et al., 2002b; Gude et al., 2005).

CONCLUSION AND SUGGESTION

The occurrence of *Arcobacter* in plant A was approximately 67% and 74% on the first and the second sampling days, respectively, while the occurrence of *Arcobacter* in plant B was 53% on both the first and the second sampling days. No significant difference between the occurrence of *Arcobacter* in plant A and plant B was observed. *A. butzleri* was the dominant species found in both processing plants. In this study, *Arcobacter* contamination in slaughterhouses tended to increase throughout slaughtering process. This finding is likely due to the accumulation of *Arcobacter* on the surface of equipment in slaughterhouses which resulted in cross-contamination from carcass to carcass. Although the application of inside/outside washing and chilling was used for reducing microorganisms, higher concentration of *Arcobacter* was found in finished products suggesting that inside/outside washing and chilling steps did not effectively reduce *Arcobacter* contamination.

In terms of genetic profiles of *Arcobacter* isolates, although the present study revealed that *Arcobacter* had a high genetic diversity, some fingerprint patterns were detected in *Arcobacter* isolated from both environmental and chicken-related samples at different slaughtering stages. These findings indicate that cross-contamination between slaughterhouse environment and chicken products along the processing line may occur via contact surface. Even though *Arcobacter* could be isolated from cloacal swab samples, poultry gut is unlikely the main route of *Arcobacter* contamination in chicken products because the isolates from cloacal swabs were genetically different from *Arcobacter* isolates recovered from chicken-related samples. Slaughterhouse environment may harbor *Arcobacter* and can lead to chicken carcass contamination. Similar *Arcobacter* genotypes were observed even the second sample collection was several weeks apart from the first sample collection. This finding indicates that some *Arcobacter* genotypes may circulate in the slaughterhouse environment and re-contaminate chicken carcasses during processing. Since the exact origin and route of carcass contamination are still unclear in the present study, further studies should focus on the source of *Arcobacte*r

contamination at the slaughterhouse level and strategies to reduce *Arcobacter* contamination in finished products.

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APPENDIX

APPENDIX A

Culture media used for *Arcobacter* isolation

1. *Arcobacter* enrichment broth (CM0965; Oxoid)

	(gm/litre)
Peptone	18.0
Yeast extract	1.0
Sodium chloride	5.0

pH 7.2 ± 0.2 @ 25°C

2. CAT selective supplement

	(mg /litre)
Cefoperazone	8.0
Amphotericin B	4.0
Teicoplanin	10.0

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

	(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

	(mg/litre)
Cefoperazone	32.0
Amphotericin B	10.0

Illustration of *Arcobacter* isolation procedure (Selective enrichment method)

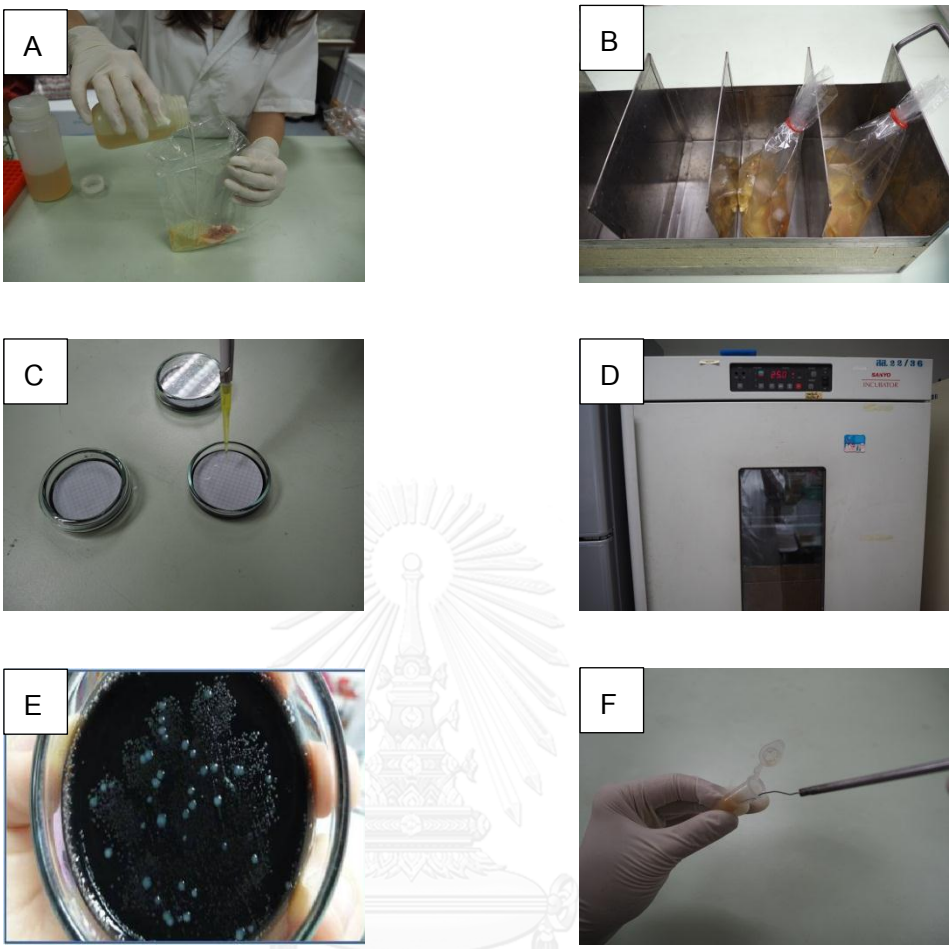

Illustration of *Arcobacter* isolation

A, Each sample was enriched in *Arcobacter* enrichment broth (AEB) supplemented with CAT;

B, All samples were incubated at 25°C for 48 hours under aerobic conditions;

C, *Arcobacter* enrichment broth were dropped on filter membrane which was laid on mCCDA plates;

D, All inoculated plates were incubated at 25°C for 48 hours under aerobic conditions;

E, After incubation, colonies of *Arcobacter* were further confirmed by multiplex PCR;

F, Each *Arcobacter* isolate was preserved in cryovial tube containing skim milk and 30% glycerol at -80 °C for further analysis.

APPENDIX B

Table B-1 Genotypes of *Arcobacter* isolated from broilers and environment of samples collected from poultry processing plant A (90% similarity value)

Subtype	No. of related strains	Source	Stage of slaughtering	Type of samples[a]	Day of sampling
A1	3	Breast comforter (2)[b]	hanging	env	2
		Shackles	hanging	env	1
A2	3	Breast comforter	hanging	env	1
		Breast comforter (2)	hanging	env	2
A3	7	Chilling water (2)	chilling	env	2
		Carcass rinse	defeathering	bird	2
		Carcass rinse	I/O washing	bird	2
		Carcass rinse (3)	chilling	bird	2
A4	14	Chilling water	chilling	env	1
		Gloves	evisceration	env	1
		Carcass rinse	scalding	bird	1
		Carcass rinse (2)	evisceration	bird	1
		Carcass rinse	I/O washing	bird	1
		Carcass rinse	chilling	bird	1
		Gloves	defeathering	env	2
		Knife	evisceration	env	2
		Carcass rinse (2)	scalding	bird	2
		Carcass rinse (2)	evisceration	bird	2
		Carcass rinse	I/O washing	bird	2
A5	1	Breast comforter	hanging	env	1
A6	7	Shackles	hanging	env	1
		Breast comforter	hanging	env	1
		Gloves	defeathering	env	1
		Gloves	I/O washing	env	1

A6	7	Knife	evisceration	env	1
		Knife	evisceration	env	2
		Fork	evisceration	env	2
A7	1	Breast comforter	hanging	env	1
A8	4	Fork	evisceration	env	1
		Fork	evisceration	env	2
		Knife	evisceration	env	2
		Carcass rinse	I/O washing	bird	2
A9	1	Shackles	evisceration	env	2
A10	2	Carcass rinse	I/O washing	bird	1
		Carcass rinse	evisceration	bird	2
A11	1	Carcass rinse	scalding	bird	2
A12	2	Cloacal swab	hanging	bird	2
		Weight	packaging	env	2
A13	1	Table	packaging	env	2
A14	4	Gloves (3)	evisceration	env	2
		Carcass rinse	scalding	bird	2
A15	5	Gloves	defeathering	env	1
		Carcass washed	defeathering	env	1
		Carcass rinse (2)	defeathering	bird	1
		Carcass rinse	I/O washing	bird	1
A16	2	Shackles	evisceration	env	2
		Carcass rinse	I/O washing	bird	2
A17	3	Chilling water	chilling	env	1
		Carcass rinse	chilling	bird	1
		Carcass rinse	defeathering	bird	2
A18	4	Shackles	evisceration	env	1
		Shackles	hanging	env	2
		Carcass rinse	evisceration	bird	2
		Carcass rinse	chilling	bird	2

A19	4	Shackles	evisceration	env	1
		Fork	evisceration	env	1
		Shackles (2)	evisceration	env	2
A20	2	Carcass rinse	evisceration	bird	1
		Carcass rinse	defeathering	bird	2
A21	1	Carcass rinse	chilling	bird	1
A22	1	Gloves	defeathering	env	2
A23	1	Gloves	defeathering	env	2
A24	2	Carcass rinse	evisceration	bird	1
		Carcass rinse	I/O washing	bird	2
A25	3	Gloves	defeathering	env	1
		Fork	evisceration	env	1
		Carcass rinse	chilling	bird	1
A26	5	Carcass washed	evisceration	env	1
		Carcass rinse (2)	I/O washing	bird	1
		Carcass rinse	chilling	bird	1
		Carcass washed	evisceration	env	2
A27	5	Shackles	evisceration	env	1
		Gloves	evisceration	env	1
		Carcass rinse	defeathering	bird	1
		Gloves	defeathering	env	2
		Weight	packaging	env	2
A28	5	Carcass rinse	defeathering	bird	1
		Shackles	hanging	env	2
		Shackles	evisceration	env	2
		Gloves	evisceration	env	2
		Carcass rinse	defeathering	bird	2
A29	1	Cloacal swab	hanging	bird	1
A30	2	Carcass rinse	defeathering	bird	1
		Carcass rinse	evisceration	bird	1
A31	1	Gloves	evisceration	env	1

A32	5	Shackles	hanging	env	2
		Fork (2)	evisceration	env	2
		Carcass washed	defeathering	env	2
		Carcass rinse	defeathering	bird	2
A33	1	Carcass rinse	chilling	bird	2
A34	1	Carcass rinse	scalding	bird	1
A35	1	Weight	packaging	env	1
A36	1	Carcass rinse	scalding	bird	1
A37	1	Tab water	evisceration	env	1
A38	1	Shackles	hanging	env	2
A39	1	Cloacal swab	hanging	bird	2
A40	1	Fork	evisceration	env	1
A41	1	Shackles	hanging	env	2
A42	1	Breast comforter	hanging	env	1

[a]env, environment; bird, chicken-related samples.

[b]number in () indicates the number of isolates.

Table B-2 Genotypes of *Arcobacter* isolated from broilers and environmental samples collected from poultry processing plant B (90% similarity value)

Subtype	No. of related strains	Source[a]	Stage of slaughtering	Type of samples[b]	Day of sampling
B1	1	Vent gun	evisceration	env	1
B2	1	Fork	evisceration	env	2
B3	1	BB	packaging	bird	2
B4	2	Chilling water	chilling	env	2
		Carcass rinse	defeathering	bird	2
B5	1	Carcass rinse	defeathering	bird	2
B6	1	Knife	evisceration	env	2
B7	1	Table	packaging	env	1
B8	1	Breast comforter	hanging	env	2
B9	1	Breast comforter	hanging	env	2
B10	8	Shackles	hanging	env	1
		Carcass rinse	scalding	bird	1
		Carcass rinse (3)[c]	defeathering	bird	1
		Carcass rinse	evisceration	bird	1
		Carcass rinse	I/O washing	bird	1
		Carcass rinse	I/O washing	bird	2
B11	2	Carcass rinse	evisceration	bird	1
		Carcass rinse	evisceration	bird	2
B12	5	Chilling water	chilling	env	1
		Shackles	hanging	env	2
		Fork	evisceration	env	2
		Carcass rinse (2)	evisceration	bird	2
B13	3	Carcass rinse	defeathering	bird	1
		Carcass rinse	evisceration	bird	1
		Carcass rinse	I/O washing	bird	1

B14	3	Carcass rinse	scalding	bird	1
		Carcass rinse	defeathering	bird	1
		Shackles	hanging	env	2
B15	1	Shackles	hanging	env	1
B16	3	Carcass rinse (2)	scalding	bird	1
		Carcass rinse	scalding	bird	2
B17	1	Shackles	hanging	env	1
B18	2	Shackles	hanging	env	2
		Carcass rinse	defeathering	bird	2
B19	1	Wing	packaging	bird	2
B20	5	Carcass rinse (2)	chilling	bird	1
		Final product (BB)	packing	bird	1
		Final product (BIL)	packing	bird	1
		Fork	evisceration	env	2
B21	2	Final product (BIL)	packing	bird	1
		Final product (wing)	packing	bird	1
B22	1	Breast comforter	hanging	env	1
B23	1	shackles	hanging	env	2
B24	1	BIL	packaging	bird	2
B25	1	BIL	packaging	bird	1
B26	2	Carcass rinse (2)	chilling	bird	1
B27	1	Knife	evisceration	env	2
B28	1	Chilling water	chilling	env	2
B29	1	Chilling water	chilling	env	2
B30	1	Wing	packaging	bird	2
B31	4	Carcass rinse	evisceration	bird	2
		Carcass rinse	I/O washing	bird	2
		Carcass rinse	chilling	bird	2
		Final product (BIL)	packing	bird	2

B32	6	Shackles	hanging	env	2
		Shackles	evisceration	env	2
		Carcass rinse	defeathering	bird	2
		Carcass rinse	evisceration	bird	2
		Carcass rinse	I/O washing	bird	2
		Final product (BB)	packing	bird	2
B33	1	Carcass rinse	scalding	bird	2
B34	1	Carcass rinse	chilling	bird	1
B35	1	Breast comforter	hanging	env	1
B36	1	Chilling water	chilling	env	1
B37	1	Shackles	hanging	env	1
B38	1	Knife	evisceration	env	2
B39	1	Knife	evisceration	env	2
B40	2	Breast comforter	hanging	env	1
		Carcass rinse	scalding	bird	1
B41	1	Vent gun	evisceration	env	2
B42	5	Breast comforter	hanging	env	1
		Chilling water	chilling	env	1
		Knife	evisceration	env	2
		Carcass rinse (2)	chilling	bird	2
B43	1	BB	packaging	bird	2
B44	1	Breast comforter	hanging	env	1
B45	2	Knives (2)	evisceration	env	2
B46	2	Final product (wing)	packing	bird	1
		Shackles	evisceration	env	2
B47	3	Final product (wing) (2)	packing	bird	1
		Final product (BIL)	packing	bird	2
B48	3	Final product (BB) (2)	packing	bird	1
		Final product (fillet)	packing	bird	1
B49	1	Fillet	packaging	bird	1

B50	2	Chilling water	chilling	env	2
		Final product (wing)	packing	bird	2
B51	1	Carcass rinse	I/O washing	bird	1
B52	1	Carcass rinse	scalding	bird	2
B53	1	Wing	packaging	bird	2
B54	1	BIL	packaging	bird	1
B55	4	Chilling water	chilling	env	1
		Carcass rinse (2)	evisceration	bird	1
		Carcass rinse	I/O washing	bird	1
B56	1	Fillet	packaging	bird	1
B57	1	Vent gun	evisceration	env	2
B58	1	Fork	evisceration	env	1
B59	1	Fillet	packaging	bird	1
B60	1	Chilling water	chilling	env	2
B61	1	Chilling water	chilling	env	1
B62	2	Shackles (2)	hanging	env	2
B63	1	Shackles	hanging	env	1
B64	1	Shackles	hanging	env	1
B65	3	Shackles	hanging	env	1
		Breast comforter	hanging	env	1
		Carcass rinse	I/O washing	bird	1
B66	1	Shackles	hanging	env	1
B67	1	Breast comforter	hanging	env	2
B68	1	Carcass rinse	scalding	bird	2

[a]BB, boneless breast; BIL, bone in leg.

[b]env, environment; bird, chicken-related samples.

[c]number in () indicates the number of isolates.

VITA

Miss Luck Hankla was born on July 4, 1986 in Chiangrai, Thailand. She got the degree in Doctor of Veterinary Medicine (D.V.M.) from the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2011. Later, she enrolled the Master of Science Program in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since academic year 2012.