CHAPTER 3

Materials and Methods

3.1 Study area and type of slaughterhouse

The study area was scoped in Chiang Mai province where it is the province that has highest poultry production in the Upper Northern part of Thailand (Thailand Information and Communication Technology Center, 2015). In this study, two types of slaughterhouse were defined to collect carcass samples, backyard and commercial slaughterhouses. Backyard slaughterhouses have slaughter capacity less than 100 head/day. The owners usually use their household area or nearby household to operate the slaughter process. Sources of chicken supplied to this type of slaughterhouse are mostly from small farms and backyard rearing and market output was focused on rural fresh markets in the community. Commercial slaughterhouses have slaughter capacity more than 10,000 head/day. This type of slaughterhouse has large investments on infrastructure and machinery followed the recommendation of good manufacturing practice (GMP) standard. Sources of chicken are mostly from big broiler companies' or contract farms that have moderate to high biosecurity. Meat products from commercial type are distributed to urban fresh markets, restaurants, hotels and companies' shops in Chiang Mai and other Upper Northern provinces.

3.2 Slaughterhouse survey and baseline information collection

Baseline surveys were made to collect descriptive information regard to general information of slaughterhouses. List of backyard slaughterhouse was obtained from a previous study in Chiang Mai (Chotinun, 2012) and a list of commercial slaughterhouses was obtained from the Chiangmai Provincial Livestock Office (2015). Both backyard and commercial slaughterhouses were selected by convenience sampling depends on approval to access for observation and collection of baseline information. Data on processing steps and management along the slaughter production line was collected in all eligible slaughterhouses. The slaughter processes of each slaughterhouse were observed and

recorded (Figure 3.1 and 3.2). Other available information was included, such as slaughter capacity (head per day), operating time, source of chickens, stunning and slaughtering methods, temperature, water source, step of washing and chilling, average weight of chicken carcasses and so on.

There were three commercial plants have been operating in Chiang Mai province with a mean slaughter capacity of 12,000 head per day (minimum 10,000 and maximum capacity 15,000 head per day). Two of the three commercial plants were eligible for sample collection. Chicken meat products have been distributed to 8 provinces in Upper Northern of Thailand included Chiang Mai, Chiang Rai, Nan, Phayao, Phrae, Mae Hong Son, Lampang, and Lamphun. From a preliminary survey, commercial plants use a similar commercial slaughter process, including electrical stunning and bleeding methods. For backyard type, the number of slaughterhouses is not officially reported. List of 33 eligible plants were acquired from an earlier survey (Chotinun, 2012) and 60 backyard slaughter plants in Chiang Mai have been estimated. In this study, four of those plants were selected to collect baseline information. Mean capacity per day was 23 (minimum 10 and maximum 40 chickens per day). The slaughter process employed by most backyard slaughterhouses is generally similar to each other though different from that used in commercial plants. Various techniques have been performed for slaughtering at backyard slaughterhouses including bleeding (throat cutting), cervical dislocation, and neck hanging, with other differences depending on the expertise of the owners.

In backyard type, the use of facilities commonly found in households was applied such as keeping carcasses in modified refrigerator packed with ice as a cooler box for chilling step, using a tub to immerse all chicken carcasses before proceeding to the next slaughter steps, and so on. In commercial slaughterhouses, there were some measures differently used in each plant such as inside-outside washing that automatic system has been performed in one slaughterhouse while the other used combined human power and machine for inside and outside carcass washing, respectively. Immersion chilling was slightly different in detail; the overflow system has been applied in one slaughterhouse and immersion of carcasses in the tank of cold water (with ice) without overflow system has been used in other slaughterhouse.

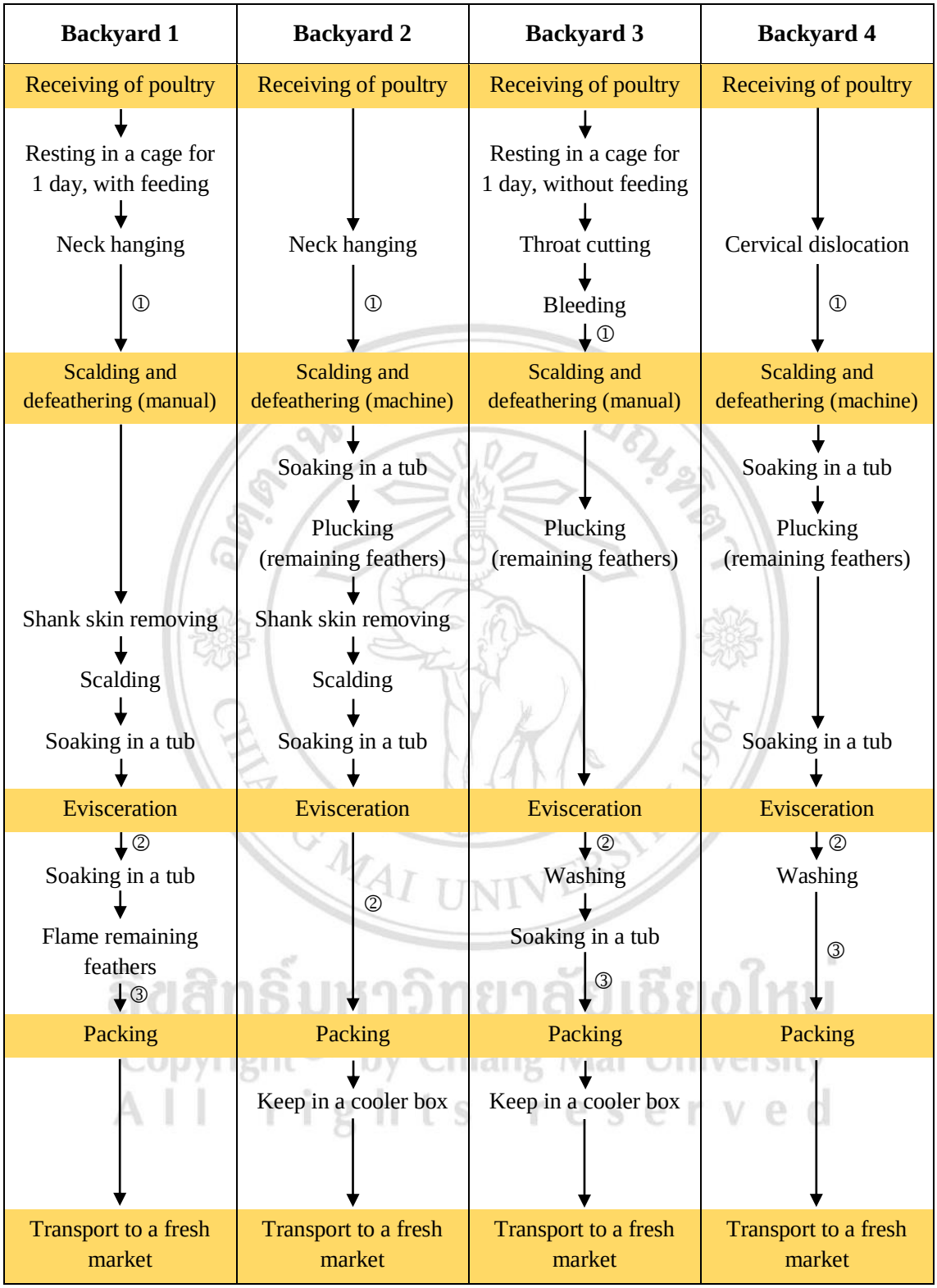

Figure 3. 1 Slaughter process of each eligible backyard slaughterhouse

Highlighting indicates the similar slaughter steps of backyard slaughterhouses.

Numbers (①,②,③) indicate the sample collection steps in this study.

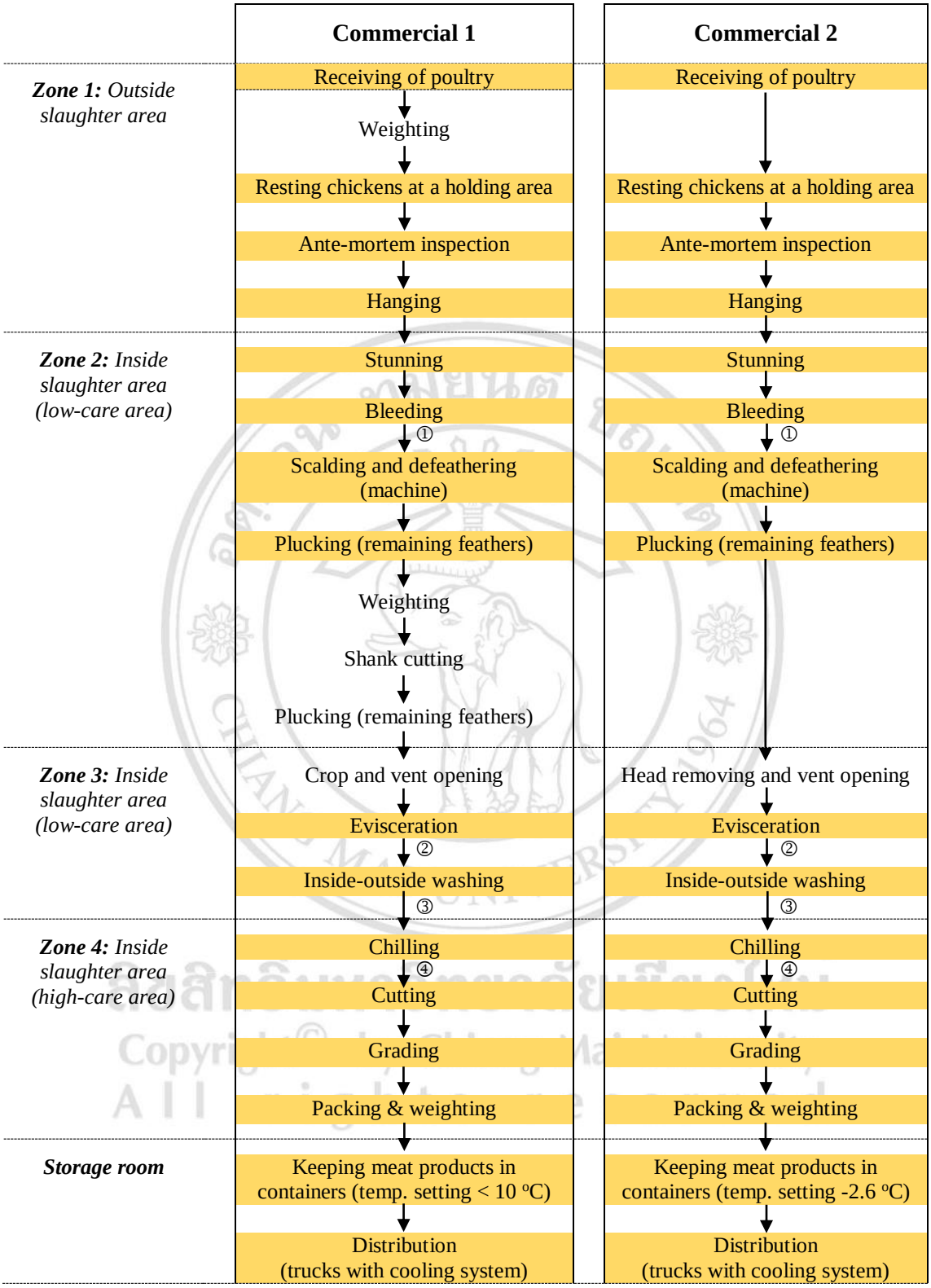

Figure 3.2 Slaughter process of each eligible commercial slaughterhouse
Highlighting indicates the similar slaughter steps of commercial slaughterhouses.
Numbers (①,②,③,④) indicate the sample collection steps in this study.

3.3 Sample size calculation

From the observation, the total slaughter capacity of commercial slaughterhouses was 31,000 head/day. Twenty-seven percent of chicken carcasses from commercial production per day, approximately 8,370 head/day, has been distributed in Chiang Mai province and the rest has been distributed to other provinces in Upper Northern Thailand (Chiang Rai, Nan, Phayao, Phrae, Mae Hong Son, Lampang, and Lamphun) (Figure 3.3). The mean slaughter capacity of backyard slaughterhouse was 18 head/day (data from 4 slaughterhouses) and approximately 60 backyard plants in Chiang Mai were estimated. So, the production of chicken carcasses from backyard type was 1,080 head/day. A total of 144 carcass samples was planned to collect from backyard and commercial slaughterhouses. The number of 108 and 36 carcass samples was designed to proportionally collect from commercial and backyard slaughterhouses, respectively, based on the proportion of the number of production per day in Chiang Mai (Table 3.1).

Table 3.1 Sample size and sample collection plan

Type of slaughterhouse	Estimated slaughter capacity (head/day)	Number of samples (N)
Commercial	8,370	108
Backyard	1,080	36
Total	9,450	144

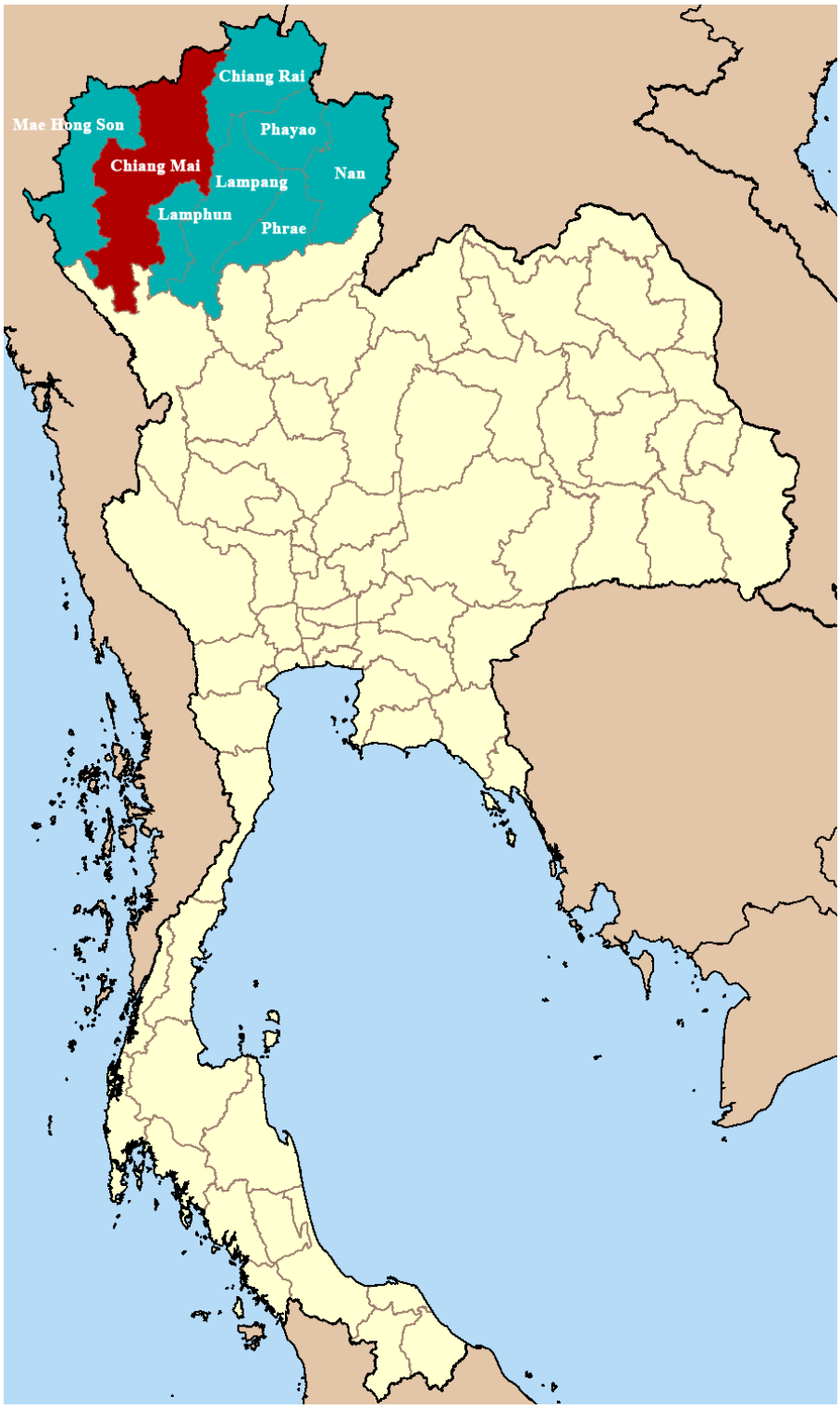

Figure 3.3 Map of Thailand

Red area indicates Chiang Mai province where commercial slaughterhouses are located. Green areas indicate Upper Northern provinces that meat products are distributed from commercial slaughterhouses in Chiang Mai.

3.4 Sample collection and preparation

Collection of samples was focused on both commercial and backyard slaughterhouses in Chiang Mai province. Selection of slaughterhouses was performed using convenience sampling of all eligible plants, with the study design to collect samples from two of the three eligible commercial slaughter plants and four backyard slaughter plants, with each plant voluntarily agreeing to study participation. Each commercial plant was sampled 3 times, based on proportion of slaughter capacity per day, while each backyard was sampled 1 time. Within each plant, selection of samples was performed by random sampling method.

A total of 105 whole chicken carcass samples was collected from both types of slaughterhouses from June to October 2016. The period of sample collection was in the rainy season (Thai Meteorological Department, 2014). The temperature ranged from 26 to 36 ^{o}C with an average of 32.2 ^{o}C, whereas total monthly precipitation ranged from 40 to 107 mm with an average of 70 mm and average humidity of 74% (World Weather Online, 2016). Thirty-three of those carcasses were randomly collected from backyard slaughterhouses along the slaughter production line at 3 different steps (Figure 3.1), 3 samples per step: slaughtering, evisceration, and final products before distributed to markets. In one backyard plant, three samples were missing from the sample plan due to no further step existed after evisceration. The remainder of the samples (n=72) were collected from commercial slaughterhouses along the slaughter production line at 4 different steps (Figure 3.2), 3 samples per step: slaughtering, evisceration, washing and chilling (assumed as final products before distributed to markets). There were 36 carcass samples missing from the sample collection plan since one slaughterhouse did not consent to collect samples. At slaughtering step, the chicken carcasses were collected to the laboratory and cecal content was collected for the test. Carcasses collected at other steps were prepared by carcass rinsing technique.

A carcass sample was collected in a sterile plastic bag. Carcass samples after collection were kept at less than 4 ^{o}C in ice boxes and transported to the Central Laboratory of the Faculty of Veterinary Medicine, Chiang Mai University within 3 hours.

3.4.1 Cecal content preparation

Ten grams of parts of cecal and small intestinal content were aseptically taken from the carcasses, collected in slaughterhouses at slaughtering step, after transport to the laboratory. Then, a 90 ml of phosphate buffered saline (PBS) (Appendix B) was added to 10 g of intestinal samples and homogenized by a stomacher for 2 min. Ten ml of the solution was added with 90 ml Bolton broth with 5% Laked Horse Blood (Oxoid®) and homogenized by a stomacher for 2 min. The initial preparation, the solution was 10^{-1}, which was 10-fold serial diluted by using PBS to 10^{-2} and 10^{-3} dilutions (Appendix C). Three dilutions of the solution were prepared for *Campylobacter jejuni* detection and enumeration as described in 3.5.

3.4.2 Carcass sample preparation

A chicken carcass was prepared in a sterile plastic bag and shaken manually in 200 ml PBS for 5 min to prepare the rinsing solution. The rinsing solution was homogenized by a stomacher for 2 min and poured into a sterile container. Then, 25 ml of the solution was added with 225 ml Bolton broth with 5% Laked Horse Blood (Oxoid®) and homogenized by a stomacher for 2 min. The initial preparation, the solution was 10^{-1}, which was 10-fold serial diluted by using PBS to 10^{-2} and 10^{-3} dilutions. Three dilutions of the solution were prepared for *C. jejuni* detection and enumeration as described in 3.5.

3.5 Detection of *Campylobacter jejuni*

3.5.1 Determination of the presence or absence of the organism

Detection of *Campylobacter jejuni* was modified from the ISO 10272-1:2006 (International Organization for Standardization, 2006) by applying the multiplex PCR for species identification instead of biochemical test. The initial solution in a sterile container was incubated in a microaerophilic atmosphere using CampyGen™ 2.5L (Thermo Scientific®) at 37 ºC for 6 h and 41.5 ºC for 44 h ± 4 h, respectively. Then, the solution was isolated onto a modified charcoal cefoperazone deoxycholate (mCCD, Oxoid®) agar. After incubation, four to five representative colonies (greyish, often with a metallic sheen, and are flat and moist, with a tendency to spread) were separately streaked onto CBA (Oxoid®) with 5% sterile defibrinated sheep blood and incubated in a microaerophilic

atmosphere at 41.5 ºC for 44 h ± 4 h. Colonies grown on CBA were preserved in 1 ml PBS for DNA extraction at the next step (Appendix C - Procedures of *Campylobacter jejuni* detection and enumeration using direct counting method).

3.5.2 Direct counting method

Drop plating procedure was performed with duplication, starting with dropping 0.1 ml of prepared dilutions onto a modified charcoal cefoperazone deoxycholate (mCCD, Oxoid®) agar. The drop was spread thoroughly on the plate until agar surface dried, then incubated in a microaerophilic atmosphere using CampyGen™ 2.5L (Thermo Scientific®) at 41.5 ºC for 44 h ± 4 h. After incubation, all colonies grown on the agar were counted and grouped based on the characteristics of 3 major groups: greyish non-spherical, spherical, and pinpoint-like. Four to five representative colonies from each group were separately streaked onto CBA (Oxoid®) with 5% sterile defibrinated sheep blood and incubated in a microaerophilic atmosphere at 41.5 ºC for 44 h ± 4 h. Colonies grown on CBA were preserved in 1 ml PBS for DNA extraction at the next step (Appendix C - Procedures of *Campylobacter jejuni* detection and enumeration using direct counting method).

3.5.3 DNA preparation

One ml of pure culture mixed with PBS (from 3.5.1 and 3.5.2) was centrifuged at 10,000 rpm for 5 min and the supernatant was removed. The pellet was resuspended in 200 µl of sterile distilled water, then centrifuged at 10,000 rpm for 5 min and the supernatant was removed. The pellet was resuspended in 500 ul distilled water. The bacterial suspension was boiled in thermoblock at 100 ºC for 10 min and refrigerated at 0 ºC for 10 min. After that it was centrifuged at 14,000 rpm for 1 min. The supernatant was placed in a new microcentrifuge tube and kept at -20 ºC until confirmed by multiplex PCR.

3.5.4 Multiplex PCR

Specific primers to *Campylobacter* 16S rRNA, *C. jejuni* and *C. coli* target genes referenced from the study of Denis et al., 1999 were used to confirm the DNA of the samples. DNA samples that tested positive for 16S rRNA and *mapA* genes were confirmed as *C. jejuni*. Selected primers and their sequences were shown in Table 3.2.

Table 3.2 Primers used in multiplex PCR (from Denis et al., 1999)

Primer	Target gene	Primer sequence (5'-3')	Amplicon size (bp)	Classification
MD16S1 MD16S2	*16s rRNA*	ATC TAA TGG CTT AAC CAT TAA AC GGA CGG TAA CTA GTT TAG TAT T	857	*Campylobacter* genus
MDmapA1 MDmapA2	*mapA*	CTA TTT TAT TTT TGA GTG CTT GTG GCT TTA TTT GCC ATT TGT TTT ATT A	589	*C. jejuni*
COL3 MDCOL2	*ceuE*	AAT TGA AAA TTG CTC CAA CTA TG TGA TTT TAT TAT TTG TAG CAG CG	462	*C. coli*

A multiplex PCR method for the specific detection of thermophilic *Campylobacter* was based on amplification and detection of 857 bp, 589 bp and 462 bp for the genus *Campylobacter*, the species *jejuni* and *coli*, respectively. A total 15 µl of the mixture containing 1.5 µl of template DNA, 2x Quick Taq HS DyeMix (TOYOBO®) (7.5 µl), 0.11 µM MD16S1 and MD16S2 primers (0.3 µl each), 0.42 µM MDmapA1, MDmapA2, COL3 and MDCOL2 primers (0.25 µl each) and 4.4 µl sterile water. The amplification reactions were performed on PTC-200 Peltier Thermal Cycler™ with the following program: one cycle at 95 ºC for 10 min, amplification consisting of 95 ºC for 30s, 59 ºC for 1.5 min, 72 ºC for 1 min, then repeat amplification for 35 cycles followed by a final extension at 72 ºC for 10 min. The temperature was held at 4 ºC until gel electrophoresis was run (Appendix C - Multiplex PCR and gel electrophoresis procedures).

Three microliters of aliquots were subjected to gel electrophoresis with 1.5% of the mixed RedSafe™ agarose gel at 110V for 30 min and viewed under UV light (302 nm) using GelMax™ Imager and Doc-It®LS Image Analysis Software version 7.1, for PCR product visualization.

3.6 Contamination rate of *Campylobacter jejuni*

DNA isolate that was positive for both *16s rRNA* and *mapA* gene was recorded and counted as a *C. jejuni*-positive sample. Contamination rate of *C. jejuni* was calculated using the formula as follows.

$$\text{Contamination rate} = \frac{\text{Number of } C.\ jejuni\text{-positive samples}}{\text{Total number of samples}} \times 100$$

Contamination of *C. jejuni* was reported in a format of percentage (%) and compared using statistical analysis.

3.7 Level of contamination of *Campylobacter jejuni*

DNA isolate that was positive for both *16s rRNA* and *mapA* gene was recorded and calculated back to the number of *Campylobacter jejuni* of each rinsing samples. Direct counting calculation will be calculated based on ISO 10272-2: 2006 as follows.

a) Estimation of number of *C. jejuni* present if the two plates contained 15-150 colonies;

N is the number of *C. jejuni* presented in the sample

$$N = \frac{\sum a}{V \times [n_1 + (0.1 \times n_2)] \times d}$$

Where

$\sum a$ is the sum of colonies that were confirmed positive for *C. jejuni* counted on the two plates of mCCDA

V is volume of inoculum applied to each plate (ml)

n_1 is the number of plates retained at the first dilution

n_2 is the number of plates retained at the second dilution

d is the dilution factor corresponding to the first dilution retained (d = 1 when the undiluted sample is used, d = 0.1 when the 10^{-1} dilution is used as the first dilution)

b) Estimation of number of *C. jejuni* present if the two plates contained less than 15 colonies;

N_E is the estimated number of *C. jejuni* present in the sample

$$N_E = \frac{\sum a}{V \times n \times d}$$

Where

$\sum a$ is the sum of colonies that were confirmed positive for *C. jejuni* counted on the two plates of mCCDA

V is volume of inoculum applied to each plate (ml)

n is the number of plates retained

d is the dilution factor corresponding to the first dilution retained

The level of *C. jejuni* contamination was shown in log CFU/g or log CFU/ml of sample in the form of mean ± SD.

3.8 Statistical analysis

Comparison of prevalences by type of slaughterhouse and sampling step was conducted using a binomial test. *Campylobacter* counts were shown in the transformed unit of log CFU/g or log CFU/ml of the sample. Comparison of level of *C. jejuni* contamination between types of slaughterhouses (backyard and commercial) was conducted using independent t-test and comparison of level of *C. jejuni* contamination in the same type of slaughterhouse at different steps was conducted using one-way ANOVA. P-values less than 0.05 were considered statistically significant.

3.9 Poultry meat safety workshop for slaughterhouse stakeholders

The workshop "Poultry Meat Safety...from Slaughterhouses to Consumers" was held on December 17, 2016 with the collaboration between the Veterinary Public Health Centre for Asia Pacific (VPHCAP) and the Excellent Center of Veterinary Public Health (ECVPH), Chiang Mai University. There were 9 participants attended the workshop who were stakeholders: quality control workers, slaughterhouse managers and owners of small- and large-scale slaughterhouses. The objectives of the workshop were;

1) To build knowledge, understanding, and awareness of poultry meat safety as well as foodborne pathogens and diseases that can be occurred in slaughtering production line.

2) To exchange practical preventive and control measures in order to prevent and control the contamination in slaughtering production line to the safe level of meat for human consumption.

The first objective was covered by keynote presentations on the theme of safety chicken meat production. An update on food security in Thailand, farms and slaughterhouse standards, and the report of laboratory confirmation related to common foodborne pathogens, including *Campylobacter jejuni*, that mostly found in the slaughter production line were included in the workshop.

The second objective of the workshop was obtained from focus group discussion. The objectives of focus group discussion were to identify critical points of contamination, practical control measures, current barriers or obstacles of slaughterhouse operation, and knowledge or supports that participants needed from the university and involved stakeholders. In this activity, participants from small- and large-scale slaughterhouses were randomly divided into two groups. Focus group discussion and brainstorming activities were followed 4 main questions;

1) What are the points of production that are at risk of contamination? and why?

2) What are the practical control measures at those points?

3) Barriers or obstacles that affect the control of pathogens?

4) What are knowledge or supports needed from the university and involved stakeholders?

After finishing the workshop, the trilingual signs (Thai, Myanmar, and Tai) provided in slaughter working areas (Figure 3.3) and the educational poster of proper handwashing (Figure 3.4) were prepared to slaughterhouse representatives to use in their workplace. Activity photos were shown in Figure 3.5.

Figure 3.4 Trilingual signs provided in slaughterhouse working areas

Upper-middle-lower row: Thai, Tai, Myanmar; 1) Wash hands before and after eating, and after using the toilet; 2) Do not eat; 3) No smoking; 4) Beware of slippery floor; 5) High care area; 6) Low care area; 7) No entry

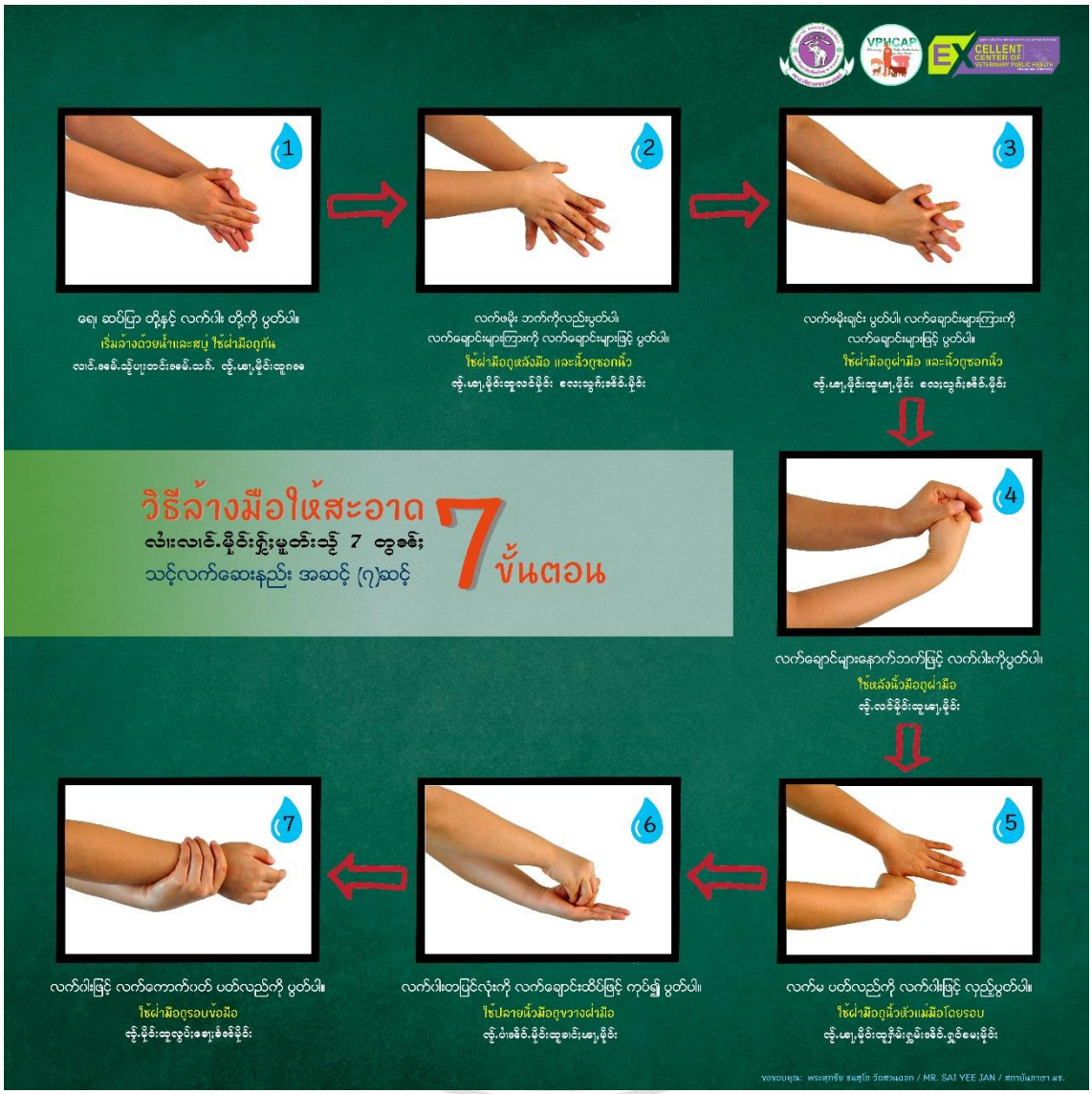

Figure 3.5 The educational poster of 7 steps proper handwashing

1) Start washing with water and soap, rub the palms together; 2) Rub the back of the hand with the palm, and using fingers to rub between fingers; 3) Rub the palms together, and using fingers to rub between fingers; 4) Rub the palm with the back of fingers; 5) Run around the thumb with the palm; 6) Using fingertips to rub across the palm; and 7) Rub around the wrist with the palm

Figure 3.6 Workshop on “Poultry Meat Safety…from Slaughterhouse to Consumers”

From left to right, top to bottom: 1) Updates on current food safety situation; 2) Presentation on laboratory testing regarding to risk factors of contamination along the slaughter line; 3) Special lecture on farms and slaughterhouse standards; 4) - 5) Focus group discussion; 6) Presentation from discussion and brainstorming session; 7) Discussion on obstacles of achieving safe meat for consumption; 8) Trilingual sings and handwashing poster presentation