3. MATERIAL AND METHODS

3.1. Study design

This study was a combined cross-sectional study design and laboratory work with an overall aim of determining the microbiological quality of pig carcasses at a slaughterhouse. Associations with some risk factors for contamination were determined using a questionnaire survey on farm management practices (Annex B) and sanitary control measures in the slaughterhouse (Annex C).

3.2. Location of study

Samples were taken from the “Dorn Du” pig slaughterhouse, microbiological work was carried out in the Bacteriology Laboratory of the National Animal Health Center (NAHC), Department of Livestock and Fisheries (DLF) in Vientiane, Lao PDR and in the Faculty of Veterinary Medicine Chiang Mai University, Thailand.

The “Dorn Du” slaughterhouse is the biggest slaughterhouse in Lao PDR at the moment. It was established in 1979. The slaughterhouse comprises two slaughter lines - one for cattle and another one for pigs. Both of them are located in the same building. The slaughtering process is carried out mostly at night. About 80-100 cattle and buffaloes and 60-80 pigs are slaughtered per day.

Slaughtering of pigs in particular includes stunning, bleeding, scalding and de-hairing, evisceration and splitting. Inspection is performed both ante-mortem on live animals and post-mortem on carcasses and visceral organs. Without chilling, the carcasses are distributed to the market immediately after slaughtering procedures have been completed.

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3.3. Study population

Target animals were all pigs slaughtered at the “Dorn Du” slaughterhouse. The particular pigs were from different farms located in the Vientiane Municipal Region and other provinces. Transportation of the animals from farms to slaughterhouse took about 1-5 hrs.

3.4. Sampling strategy

3.4.1. Sample size estimation

The sample size was calculated using the computer program “Winepiscope 2.0” by the formula given as below:

$$n = \frac{1.96^2\, p(1-p)}{d^2}$$

Because the actual *Salmonella* prevalence in Laos was unknown, for sample calculation the prevalence (p) of *Salmonella* was estimated p=50%, the accepted absolute error or precision being 5% (d=0.05) and level of confidence at 95%(t=1.96), therefore:

$$n = \frac{1.96^2 * 0.5(1-0.5)}{(0.05)^2} = 385$$

In principle, the sample size should be 385 pigs, but in this slaughterhouse only 60 -80 (average 70) pigs are slaughtered per day, and according to this, the sample size is calculated by the formula:

$$n_{adj.} = \frac{N*n}{N+n} = \frac{385*70}{385+70} = 60$$

Where: n_{adj} = adjusted sample size.

In this study 62 carcasses were sampled.

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3.4.2. Type of samples and laboratory analysis

From each pig three samples were collected and microbiological analysis as shown in Table 3.1 was performed.

Table 3.1: Type of sample and laboratory analysis

Type of sample	Laboratory analysis
Carcass swab 1*	Aerobic plate count, *Enterobacteriaceae* count and *Salmonella* isolation
Mesenteric lymph nodes	*Salmonella* isolation
Carcass swab 2**	Aerobic plate count, *Enterobacteriaceae* count and *Salmonella* isolation

* Collected after de-hairing of the pig

** Collected at the end of the slaughtering process.

3.4.3. Sampling method

The random sampling method was performed. Pig carcasses were randomly selected during the slaughtering process. Because of the laboratory capacity to analyze only a limited number of samples per day, the number of samples per day was not more than 20 samples. According to this, up to seven carcasses per sampling day were sampled.

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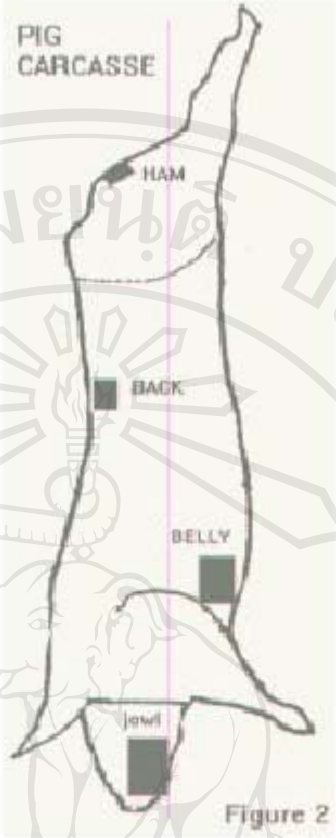

Figure 3.1: Location for swab sampling
Source: Commission decision 2001/471/EC.

3.4.4. Samplecollection

Swab samples

The cotton wool swabs were used, as described by Van den Elzen and Snijders (1993), Palumbo *et al.* (1999) and Byrne *et al.* (2005), and which were validated for *Salmonella* isolation by Swanenburg (2000). Swabs were moistened with buffered peptone water (BPW) and rubbed initially vertically, then horizontally, then diagonally across the entire surface delineated by a sterile template. Each carcass was swabbed 4 sites (from the back, jowl (or cheek), hind limb medial (ham), and belly (Figure 3.1) with one swab per sampling site. The sampling area for swabs was 10 cm by10 cm, which covered 100 $cm^{2;}$ total area swabbed was 400 cm^2 per carcass.

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Mesenteric lymph nodes

Mesenteric lymph nodes were collected immediately after evisceration of the pig in a separate room beside the slaughter line by excision using sterile scissors and forceps. About 25 g of mesenteric lymph node were cut out and kept in a plastic bag.

The samples were stored in a cool box and transported to the laboratory, where after the microbiological procedures were performed the same day.

3.5. Microbiological analysis

3.5.1. Preparation of first macerate

Swab samples were homogenized in plastic bags (Stomacher bag) for at least two minutes in 100 ml of buffered peptone water at about 250 cycles of a peristaltic Stomacher. Thereafter serials of ten-fold dilution for Aerobic plate counts and *Enterobacteriaceae* counts were prepared.

Mesenteric lymph nodes were put into boiling water for 3 seconds to eliminate superficial contamination (Swanenburg *et al.* (2001), and thereafter-cut into small pieces with sterile materials. Thereafter, 25g were transferred into a stomacher bag, and 225 ml of BPW were added and homogenized at about 250 cycles per minute for two minutes.

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3.5.2. Aerobic plate count

In general the aerobic plate count is designed to detect an estimate of the total number of aerobic organisms in a particular sample. There are two methods available for Aerobic plate counts, namely: Surface inoculation (Spread Plate) and Pour-plate procedures (David *et al.*, 1995).

In this study the pour-plate procedure was performed. A series of dilutions (10^{-2}, 10^{-3} and 10^{-4}) of the sample homogenate was mixed with an agar medium and incubated at 35 ºC for 24-48 hrs. Calculation of APC was done as follows:

$$N = \frac{\Sigma C}{[(1 * n_1) + (0.1 * n_2)] * (d)}$$

Where N = Number of colonies per ml or g of product

ΣC = Sum of all colonies on all plates counted

n_1 = Number of plates in first dilution counted

n_2 = Number of plates in second dilution counted

d = Dilution from which the first counts were obtained

3.5.3. Enumeration of Enterobacteriaceae.

Enumeration of *Enterobacteriaceae* followed the guidelines given in the standard operating Procedure "Enumeration of *Enterobacteriaceae* by the colony count technique" issued by the Health Protection Agency UK (2003). The method involves inoculation and confirmation.

Inoculation and incubation

Transfer 1 ml of each decimal dilution to a sterile Petri dish. Pour about 15 ml of molten violet red bile glucose agar (VRBGA), tempered in a 45°C water bath, into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the time when the medium is poured shall not exceed 15 minutes. Carefully mix the inoculums with the medium and allow the mixture to solidify. Invert the prepared dishes and place in an incubator at 37°C for 24 hours.

Counting of colonies

Colonies of *Enterobacteriaceae* produce purple red colonies with a diameter of 0.5 mm or greater and sometimes surrounded by a red zone of precipitated bile. Count

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and record the characteristic *Enterobacteriaceae* colonies on each plate containing not more than 150 colonies. Above this number it is likely that colonies will have an atypical appearance. The number of *Enterobacteriaceae* was calculated the same as the calculation of the Aerobic plate count.

Confirmatory tests

Subculture five suspect *Enterobacteriaceae* colonies onto a segment of a nutrient agar (NA) plate and incubate at 37^{o}C for 24 +2 hours. Use the growth obtained for biochemical confirmation.

Oxidase test

Prepare a fresh solution of the reagent for each time of use. Immerse a swab in oxidase reagent and touch lightly to the surface of the colony to be tested. The immediate appearance of a dark purple colour at the point of contact denotes a positive reaction but no colour change or a purplish colour which develops later are both negative reactions.

Fermentation test

Prior to use, steam or boil the glucose agar for 10 minutes and allow to set.

Perform a fermentation test on oxidase negative subcultures by a deep stab inoculation of tubes of glucose agar and place in an incubator at 37^{o}C for 24 +2 hours. *Enterobacteriaceae* produce a yellow colour throughout the medium.

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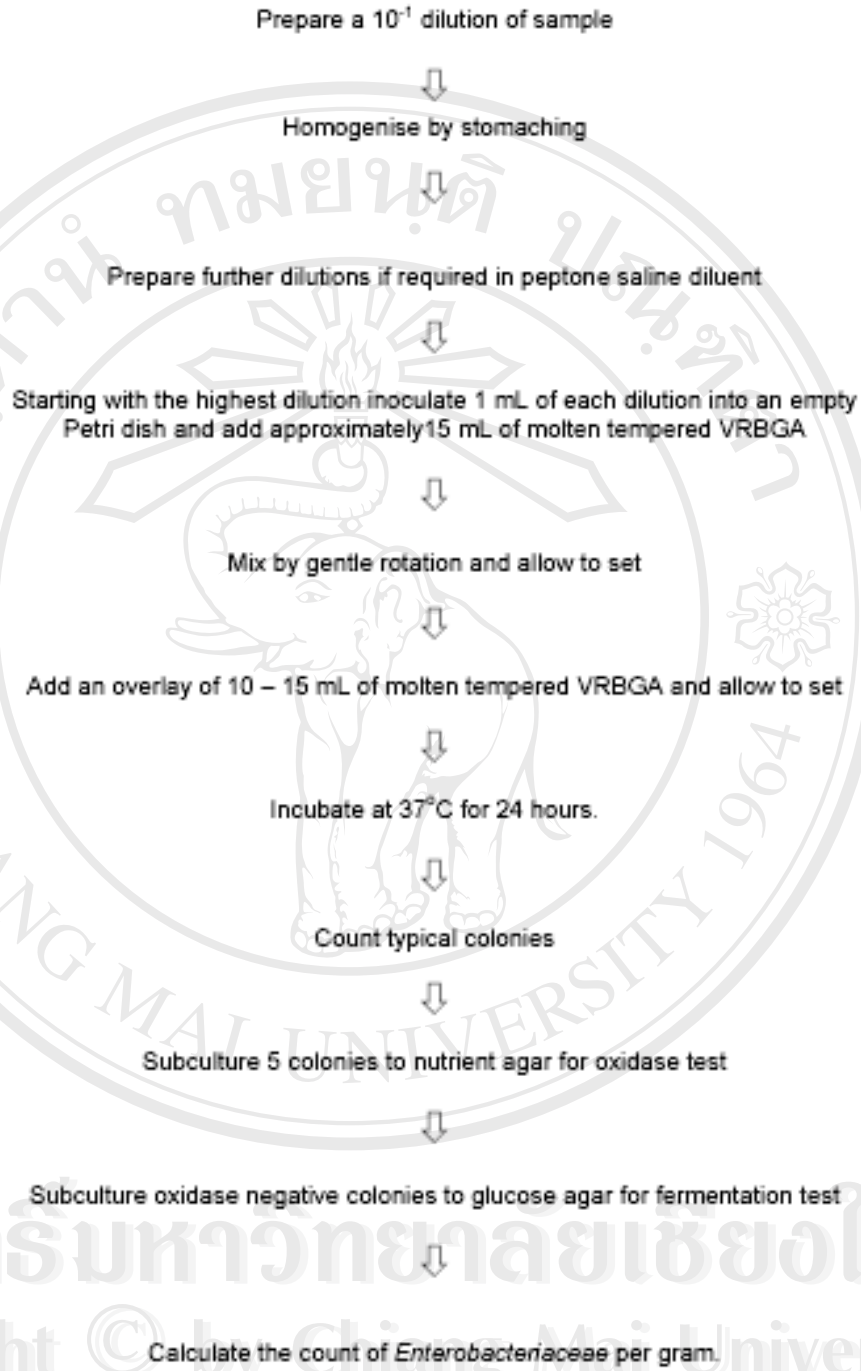

Figure 3.2: Flowchart of the enumeration of *Enterobacteriaceae* by the colony counts technique

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3.5.4. *Salmonella* isolation and identification

The conventional method for *Salmonella* isolation and identification followed guidelines given from the Institute of Meat Hygiene, Freie Universitat Berlin (July, 2004) and ISO 6579-2002. The procedure consists of the following steps.

Non-Selective Enrichment (Pre-enrichment).

The test samples were initially inoculated into a non-inhibitory liquid medium to favor the repair and growth of stressed or sub-lethally injured salmonellae. The required volume of analytical unit was dispersed into nonselective enrichment broth (BPW). Incubate the pre-enrichment mixture at 35±0.5^{o}C for 18-24 hrs.

Selective Enrichment:

Replicate portions of each pre-enrichment culture are inoculated into Muller-Kauffmann tetrationate broth and Rappaport-Vassilidiadis (RV) to favor the proliferation of salmonellae through a selective repression or inhibition of the growth of competing microorganisms, according to ISO 6579: 2002.

Selective plating:

A loopful from each selective enriched culture was streaked onto XLD and BPLS agar plates. The plates were incubated at 37^{o}C for 24 hrs. Five suspected colonies having the typical appearance of *Salmonella* were transferred to nutrient agar plates and incubated at 37^{o}C for a further 18-24 hrs. (Manufacture of all media used in this study: Merck, Darmstadt, Germany.

Serological Identification:

The serotyping consisted of the determination of the O (somatic), H (flagella with biphasic strain) and Vi (capsular if necessary) antigens according to the Kauffmann-White Scheme. Polyvalent and/or somatic grouping antisera were initially used for preliminary identification of isolates as members of the genus *Salmonella*. Secondly, the isolates were screened with Enteroclon Anti-*Salmonella* polispecific A-E and Enteroclon Anti-*Salmonella* polyspecific F-67, and thirdly, determination of the

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O group by means of group-specific Enterocolns was performed. These antisera were produced by the *SIFIN* Company, Berlin, Germany.

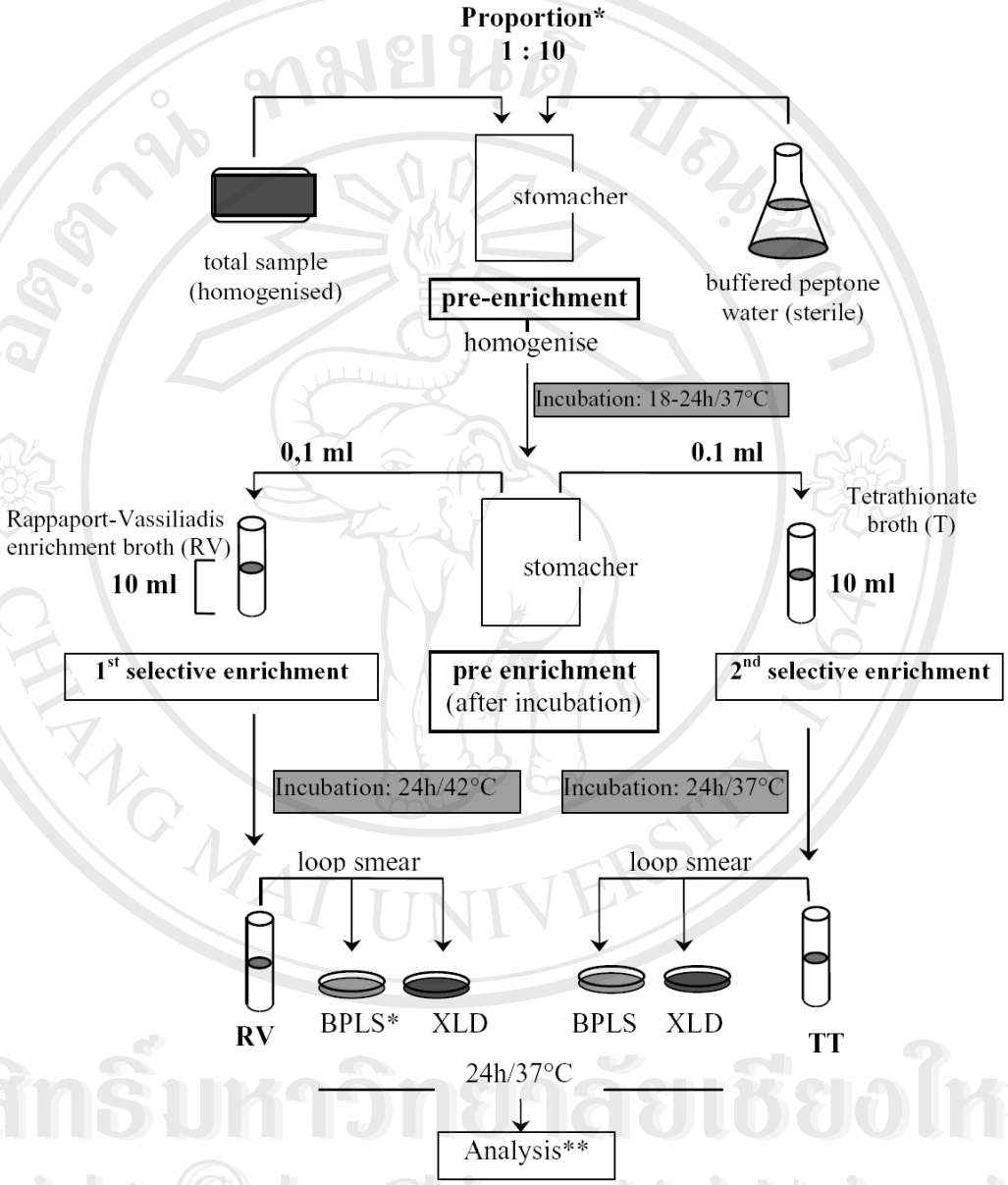

Figure 3.3: *Salmonella* Isolation and Identification Flow Chart

* Swab sample 400 cm^2 in 100 ml of BPW; Mesenteric Lymph Nodes 25g with 225 ml BPW

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** In case of finding suspicious colonies on the selective media, a biochemical and serological confirmation must follow.

- for biochemical confirmation 5 cfu, testing for pure culture on standard media, followed by biochemical testing.
- for serological confirmation 5 colonies are used for agglutination test with polyvalent serum I/II and III

3.6. Data management and analysis

The data were managed in Microsoft Excel and NCSS-PASS (Dawson). Analysis and calculation of sample-specific prevalence of S*almonella* and their differences were performed using the computer program EpiCalc version 2000. Testing for differences between two means was performed to compare the difference between the Aerobic Plate counts and the *Enterobacteriacea*e Counts of the swab samples collected from carcasses before and after evisceration of the pigs. Multivariate (Binary Logistic Regression) testing was performed for *Salmonella* prevalence among samples, and univariate analyses of the potential risk factors and occurrence of *Salmonella* in mesenteric lymph nodes were performed by using Statistics program MINITAB-13.

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