3. MATERIAL AND METHODS

Three separate studies on the prevalence of *Salmonella* in the pork production chain were performed. Sampling was carried out from the same pigs studied at farm level, prior to slaughter, and during the slaughtering process.

3.1 Place of investigation and data collection

This study was performed at a slaughterhouse and retail markets (supermarkets) in the Chiang Mai Province, Thailand. The slaughterhouse handled approximately eighty pigs per day from farms located mainly in the Chiang Mai and Lamphun provinces in the northern part of Thailand.

3.2 Materials for *Salmonella* determination

Collection of samples started after the pork carcasses were handled removed from the slaughter chilling room into the cutting room. In this room, carcasses were cut by staff members and each respective carcass was termed "**cut pork**". Some portions from the same carcasses were sent to the packaging unit of the slaughterhouse, packed into containers and transported by a slaughterhouse truck to the laboratory. These were called "**transported pork**".

Retail pork from the same batches of pork parts, such as bones, bellies, ribs, collars, loins, ground pork, shoulder meats, hams, and fillets were sent to meat departments in supermarkets. Some of these were sampled, bought and taken to the laboratory for analysis.

Environmental samples were taken in the slaughterhouse the same day as the pigs arrived in the slaughterhouse. The samples taken from surfaces of cutting boards, plastic curtains, knives, shackles, and staff hands were examined.

3.3 Collection of pork samples

3.3.1 Cut pork samples

Samples from each carcass, already individually identified at the farm were collected in the cutting room prior to packaging. Five samples were collected, i.e. belly, fillet, neck, shoulder and loin (Appendix D). These pieces called "cut pork" were combined in the same package for analysis. Sampling started at the beginning of the day (8.30-9.00 am.). The temperature of the carcasses and room temperatures were recorded (Figure 6).

3.3.2 Transported pork samples

After cutting processing, each part of the individually identified pork such as the neck of the identified carcass was packed in the same plastic bag, from the cutting unit, therefore, there were 5 packs of samples which were already sampled and packaged at the packaging unit of the slaughterhouse. They were kept in the chilling room for 1-2 days and they were transported to the faculty of Veterinary Medicine, Chiang Mai University by slaughterhouse truck, and called "transported pork". In the laboratory, 5 parts of each pig were recombined to be one sample to obtained (Figure 6).

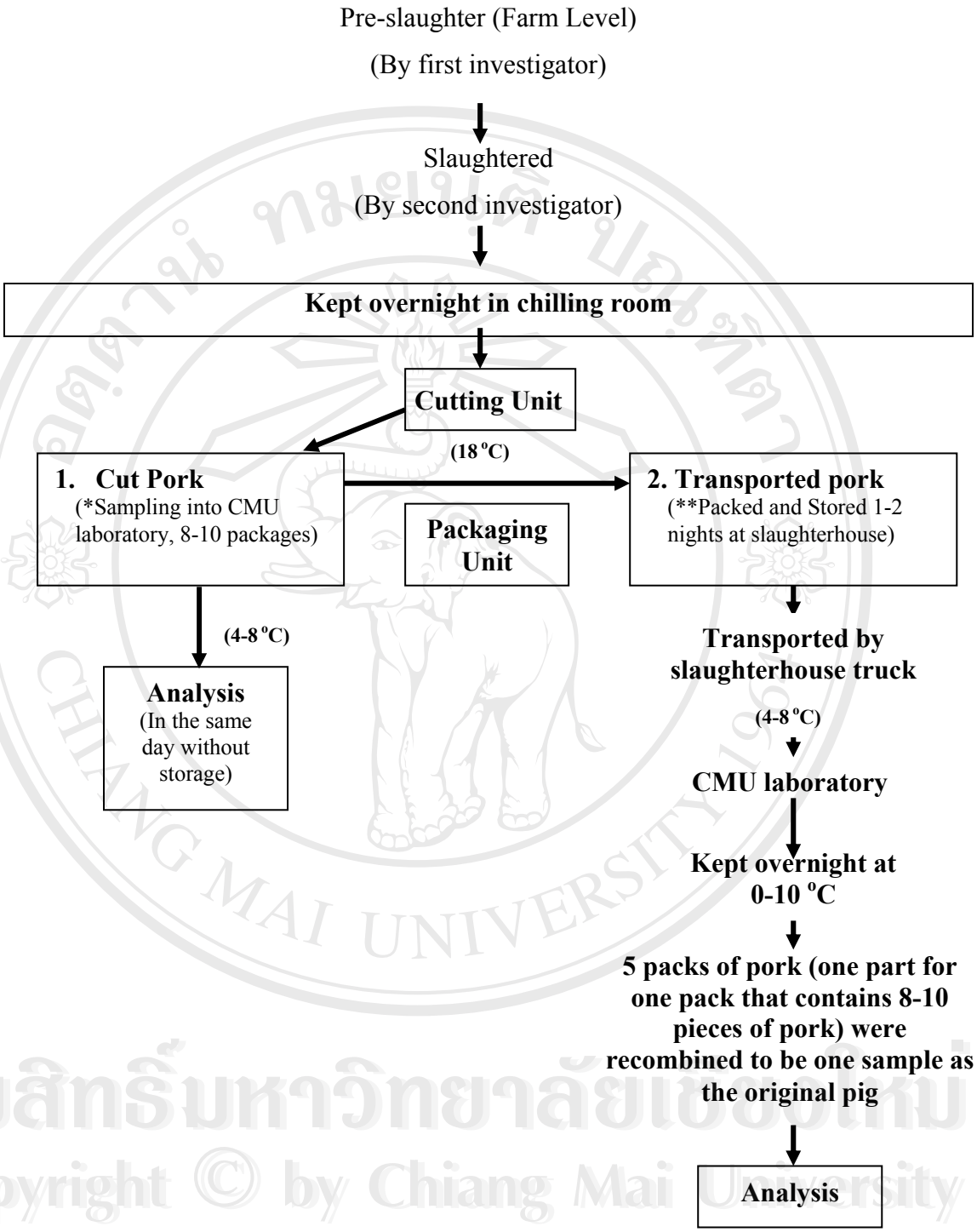

Figure 6: Schematic representation of "cut pork" and "transported pork" products

1)* 8-10 pigs = 8-10 pork samples, each pig collecting 5 parts: belly, loin, shoulder, fillet and neck. 5 parts of each pig combined in the same package

2)**The same 10 pigs as in 1)* ,each part of carcass such as neck of all 10 pigs were packed in the same plastic bag. Therefore, there were 5 packs of samples. Then, they were kept them in a chilling room 1-2 days and after that sent to the CMU laboratory. In the laboratory, 5 parts of each pig were combined to be one sample (10 samples from 10 pigs).

3.3.3 Retail pork samples

For retail products, identified packaged pork was transported from the slaughterhouse to supermarkets. Samples such as bones, bellies ribs, collars, loins, ground pork, shoulder meats, hams, and fillets from the same batch as the cut and transported pork products were conveniently sampled, taken from supermarkets on the last day after cold storage at -1 to 2^{o}C for 2-3 days. The schematic floor diagram of retail sample collection is shown in Figure 7.

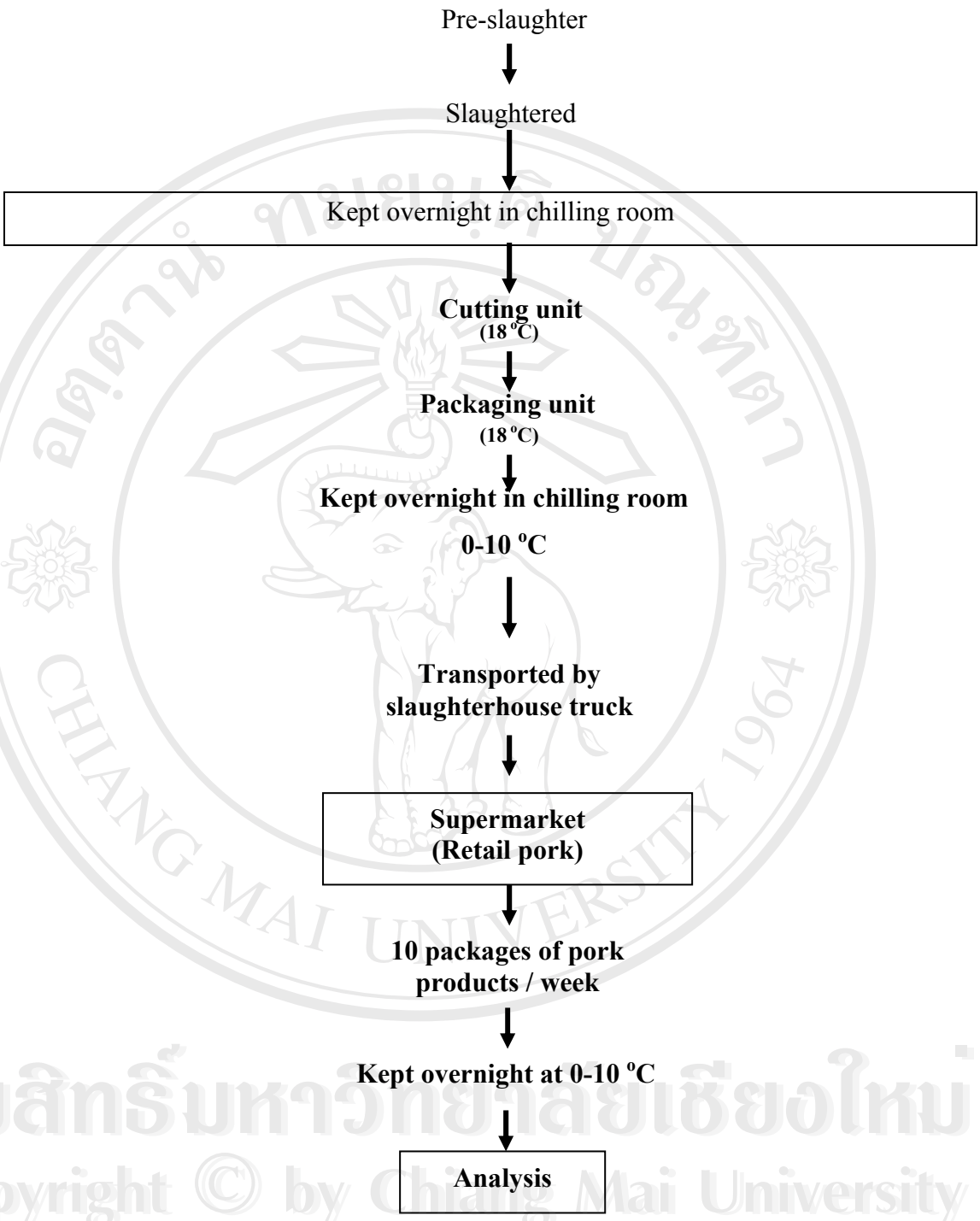

Figure 7: Schematic flow diagrams of convenient sampling of retail pork products from supermarkets

3.4 Collection of Environmental samples

Samples from equipment (cutting boards, plastic curtains, knives, shackles, hands of staff) were collected by a non–destructive method (swab technique) as described in section 3.6. There were 3 time intervals for environmental sample collection in the slaughterhouse: (1) prior to cutting (8.00-8.30); the first swab environmental samples were collected from cutting boards, plastic curtain, knife, shackles and hands of staff, called "**before cutting operation**"; (2) During cutting (8.30-11.00), the second swab samples were collected from cutting boards, plastic curtain, knife shackle and hands of staff again, called "**during cutting operation**"; (3) the third swab samples at the same position were done after cleaning and disinfecting during a lunch break (11.00-12.00), called "**after disinfecting operation**". The distribution of environmental samples collected at 3 intervals in the cutting unit of the slaughterhouse per day is shown in Table 5.

Table 5: Collection of environmental samples at 3 intervals per day in the slaughterhouse

Sampling materials	**Before cutting operation**	**During cutting operation**	**After disinfecting operation**	**Total**
Swab technique	**Number of samples**			
Cutting board	1	1	1	3
Plastic curtain	1	1	1	3
Knife	1	1	1	3
Shackle	1	1	1	3
Hands of staff	1	1	1	3
Total				15[a]

[a] Total number of swabbing samples per day

3.5 Sample size determination

The sample size was calculated by Win Episcope software (Win Episcope®, Version 2.0, 1998). Estimation of numbers of samples was based on an assumption of the prevalence of *Salmonella* infection in pre-slaughter pigs in the Chiang Mai province of 69.5% (ranging from 50-83%) (Patchanee, 2002), with a 95% confidence interval, and with an accepted error rate of 8%. All together 346 samples, from 20 farms, were collected from the slaughterhouse (173 samples from "cut pork" and 173 samples from "transported pork").

For retail, all samples were collected from one supermarket in the Chiang Mai province on one occasion. Ten samples of several types of products were collected from supermarkets. All sampling took place between January–May 2005. In total, 200 pieces of retail pork samples were collected.

A total of 300 environmental swabs in the slaughterhouse were collected.

3.6 Methods of sample preparation

3.6.1 Destructive method

From one pig, five pieces of tissues totaling 25 g were collected from five parts, pooled and put into sterile plastic bags (Stomacher). In the latter, 225 ml of sterile non-selective pre-enrichment medium was added (Buffered peptone water) at ambient temperature and sent to the laboratory unit in Chiang Mai University.

Wrappings from samples in packages from retail markets were removed carefully without touching the pork. Sterile forceps were used for putting 25g of samples into sterile plastic bags. Another 225 ml buffered peptone water was used.

3.6.2 Non-destructive method (swab technique)

The swab samples were taken from the surface of the environmental samples. The cotton swabs were wrapped in aluminum foil and were sterilized for 15 minutes

at 121 ^{o}C before use. The swabs were held in sterile forceps and the surfaces were swabbed 10 times from top to bottom carefully applying firm pressure on every surface (Table 5) according to the EU decision 2001/471. Prior to sampling, the swab was moistened with normal saline solution. After swabbing the surfaces, the swabs were put into 50 ml of sterile buffered peptone water in a plastic bag and shaken by hand for 2 minutes. The fluid was kept in an ice box (4-5 ^{o}C). All samples were forwarded to the laboratory for analysis.

3.7 *Salmonella* isolation procedures

In this study, conventional methods for the detection of *Salmonella* were carried out, following ISO 6579:2002 (Microbiology of food and animal feeding stuffs-horizontal method for the detection of *Salmonella* spp.) The Diagram of the procedure for the detection of *Salmonella* spp. is given in Figure 8.

3.7.1 Non-selective pre-enrichment

Using the non-destructive technique, a swab sample (cotton swab) was put into 50 ml of buffer peptone water (BPW). With the destructive method, 25 g of pork was transferred into a stomacher bag with 225 ml BPW. They were shaken in a stomacher for 2 min. All samples were incubated at 37 ^{o}C ± 1 ^{o}C for 18 ± 2 h.

3.7.2 Selective enrichment

The pre-enrichment broth was mixed and 0.1 ml was transferred into a tube containing 10 ml of RVS broth (Rappaport-Vassiliadis medium with soya). For the 2nd environment, 1 ml of the pre-enrichment broth was transferred into a tube containing 10 ml of MKTTn broth (Muller-Kauffmann tetrathionate novobiocin broth).

The inoculated RVS broth was incubated at 41.5 ^{o}C±1 ^{o}C for 24 h ± 3 h and the inoculated MKTTn broth at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

3.7.3 Plating and identification

After incubation for 24 h ± 3 h, a loop of material from RVS broth and MKTTn was transferred and streaked separately onto the surface of XLD agar (Xylose lysine deoxycholate agar) and BPLS agar (Brilliant green Phenol Red Lactose Sucrose agar) separately. The plates were incubated in an inverted position at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h. After incubation, the plate was checked for growth of typical *Salmonella* colonies.

Typical colonies of *Salmonella* grow on XLD agar with a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator (*Salmonella* H_2S negative variants (e.g. *S.* Paratyphi A). On XLD agar typical colonies are pink with a darker centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening).

Typical colonies of *Salmonella* grow on BPLS agar a have a reddish color and translucent colony.

3.7.4 Confirmation

Five typical colonies per plate grown on the XLD agar and BPLS agar were transferred and inoculated on triple sugar iron agar (TSI), incubated at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

If fewer than five typical or suspected colonies per Petri dish were observed, all suspected colonies were streaked on the surface of pre-dried nutrient agar plates, in a manner which allowed well-isolated colonies to develop. The inoculated plates were incubated at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

Pure cultures were used for biochemical and serological confirmation.

3.7.4.1 Biochemical confirmation

3.7.4.1.1 TSI agar

Streak the agar slant surface and stab the butt. Incubate at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h. Interpret the changes in medium as follows.

Table 6: Meaning of TSI agar for *Salmonella* spp.

Area of Reaction	Result	Meaning
Butt		
	Yellow	glucose positive (glucose used)
	Red (unchanged)	glucose negative (glucose not used)
	Black	formation of hydrogen sulfide
	Bubbles or cracks	gas formation from glucose
Slant surface		
	Yellow	lactose and/or sucrose positive
	Red(unchanged)	lactose and sucrose negative

Typical *Salmonella* cultures show alkaline (red) slant and acid (yellow) butts with gas formation (bubble) and (in about 90% of the cases) formation of hydrogen sulfide (blackening of the agar).

3.7.4.1.2 Urea agar

Streak the agar slant surface. Incubate at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h. and examine at intervals.

If the reaction is positive, splitting of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later to deep cerise (a moderate red). The reaction is often apparent after 2 h to 4 h.

3.7.4.1.3 L-Lysine decarboxylation medium

Inoculate just below the surface of the liquid medium. Incubate at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

Turbidity and a purple colour after incubation indicate a positive reaction. A yellow colour indicates a negative reaction.

3.7.4.1.4 Detection of β-galactosidase

Suspend a loopful of the suspected colony in a tube containing 0.25 ml of the saline solution.

Add one drop of toluene and shake the tube. Put the tube in the water bath set at 37 ^{o}C and leave for several minutes (approximately 5 min). Add 0.25 ml of the β-galactosidase reagent for detection of β-galactosidase and mix.

Replace the tube in the water bath set at 37 ^{o}C and leave for 24 h ± 3 h, examining the tube at intervals.

A yellow colour indicates a positive reaction. The reaction is often apparent after 20 min.

3.7.4.1.5 Medium for Voges-Proskaur (VP) reaction

Suspend a loopful of the suspected colony in a sterile tube containing 3 ml of the VP medium (7g of peptone, 5g of glucose and 5g of dipotassium hydrogen phosphate in 1000 ml water). Incubate at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h.

After incubation, add two drops of the creatine solution, three drops of ethanolic solution of 1-naphthol and then two drops of the potassium hydroxide solution; shake after the addition of each reagent.

The formation of a pink to bright red colour within 15 min indicates a positive reaction.

3.7.4.1.6 Medium for indole reaction

Inoculate a tube containing 5 ml of the tryptone/ tryptophan medium with the suspected colony. Incubate at 37 ^{o}C±1 ^{o}C for 24 h ± 3 h. After incubation, add 1ml of the Kovacs reagent. The formation of red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

The interpretation of biochemical test for detection of *Salmonella* spp. is shown in table 7, the whole procedure in Figure 8.

3.7.4.2 Serological confirmation and Serotyping

The presence of *Salmonella* O-, Vi- and H-antigens was tested using slide agglutination reaction with the appropriate anti-sera, from pure colonies and after elimination of auto-agglutinationable strains. For agglutination testing, the prescription of the manufacturer of the antiserum was used following SIFIN (Institut für Immunpräparate und Nährmedien GmbH) and the serotyping *Salmonella* procedure of the Institute of Meat Hygiene and Technology, Freie Universität Berlin, Germany, described in Appendix B.

Table 7: Interpretation of biochemical test for salmonellae

Biochemical Test	Reaction
TSI acid from glucose (+gas formation)	Positive
TSI acid from lactose	Negative
TSI acid from sucrose	Negative
TSI hydrogen sulfide produced	Positive
Urea hydrolysis	Negative
Lysine decarboxylation	Positive
β-galactosidase reaction	Negative
Voges-Prokauer reaction	Negative
Production of indole	Negative

3.8 Data management and analysis

Program Excel version 2003 (Microsoft® Office Excel 2003, Microsoft Office Professional Edition, 2003) and NCSS statistical software (Hintze, 2001) were used for collection, management and analysis of the data. Descriptive statistics were used to describe the result for prevalence analysis. The prevalence ratio (PR) was used in this study to measure association of cut and transported pork by using the Win Episcope program (Win Episcope®, Version 2.0, 1998). The value of PR indicates; (1) PR equal to 1 means no association exists between occurrence of disease and exposure; (2) PR less than 1 means the exposure is positively associated to the disease (risk factor); (3) PR more than 1 means the exposure is negatively associated to the disease (preventive factor).

		Result or Disease		
		+	-	
Factor	+	A	B	A+B
	-	C	D	C+D
		A+C	B+D	N

$$PR = \frac{\frac{A}{A+B}}{\frac{C}{C+D}};$$

A = Number of result both were positive
B = Number of result in first step (cut) was positive and in second step (transported) was negative
C = Number of result in first step was negative and in second step was positive
D = Number of result both were negative

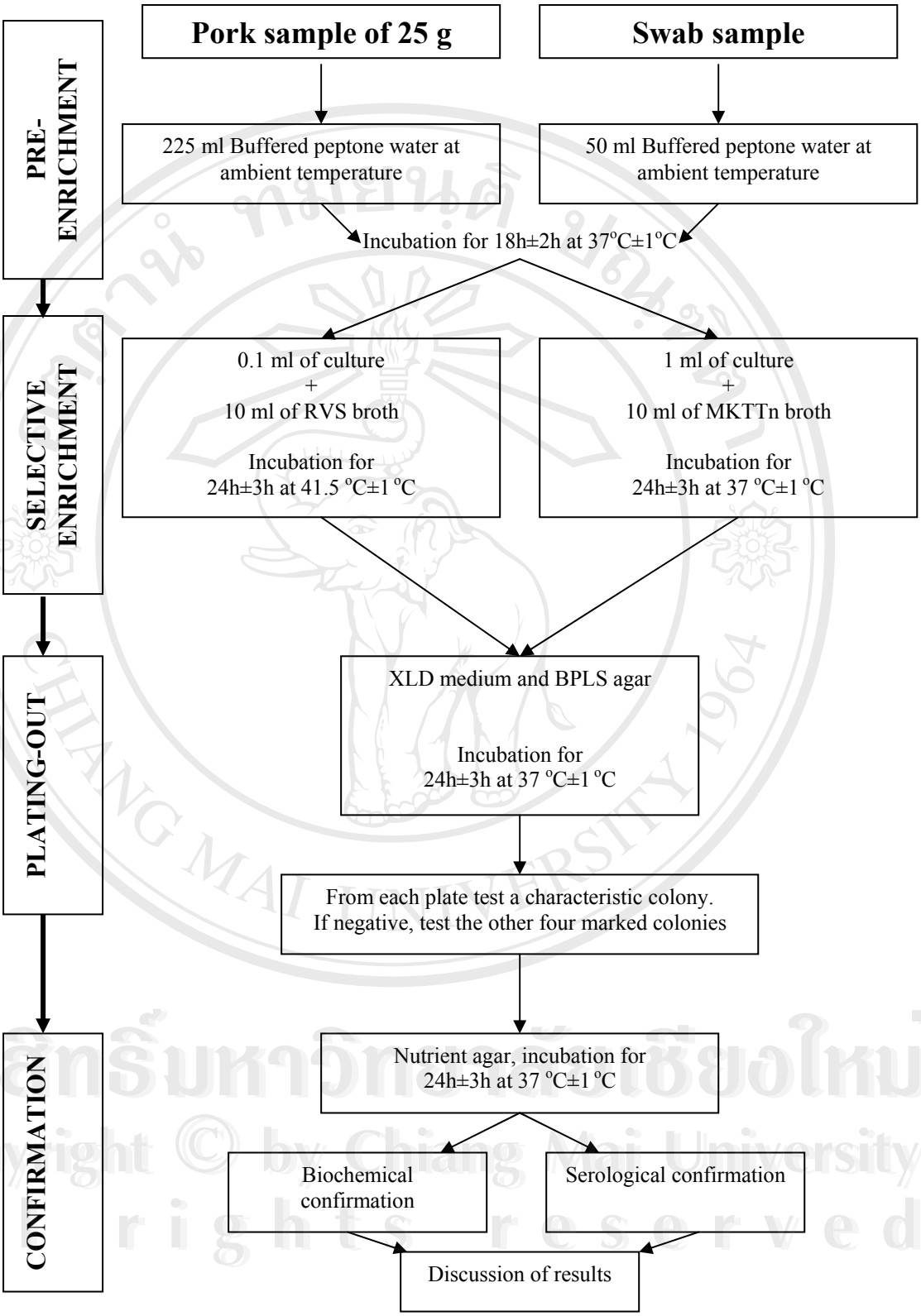

Figure 8: Diagram of procedure for detection of *Salmonella* spp.