3. MATERIAL AND METHODS

3.1 Study Design

Base on experience from recent studies, the model consisted of 2 research studies:

-the cross-sectional and prospective study to determine the prevalence of *Salmonella* in breeder sow and potential risk factors

-the cross-sectional and prospective study to determine the incidence of *Salmonella* in piglet and in the fattening period. Samples were collected during January – May 2007.

3.2 Sample Size and Sample selection

3.2.1. Sample size determination

In Thailand, the study of prevalence of Salmonella in breeder sows and their piglets had previously never been undertaken. In order to complete pork chain project as mentioned above, the estimation of the prevalence of *Salmonella* infections in piglets in Chiang Mai province had to be done by using the prevalence at fattening farm level 62.9 % (Dorn-in, 2005) with a maximum allowable error of 5% and 95% confidence level. The piglets those were delivered from 20 sows during the studying period were included in this project. Three piglets from 1 sow were identified and samples were collected from only 2 piglets. The piglets were followed until they reached an age of 120 days. The number of piglets would be enough to estimate the prevalence of *Salmonella* in piglets and in the fattening period. Environmental samples related to the risk of introducing *Salmonella* into the herd, including house floor, feed and water supply, were collected and tested for the presence of *Salmonella.*

3.2.2. Farm and pig selection

One breeder farm was selected by convenience sampling technique to represent private company that supplied weaned piglets to fattening farms in Chiang Mai, Thailand. The selected farm had 5,000 sows and separated them into two housing system, open and evaporation cooling system within the same management.

Seven days prior to delivery, the sows were moved to the cleaned and disinfected farrowing units. Each house (open and evaporation) contained 30-40 sows. In the farrowing unit sows were treated with antibiotics 2 days after delivery in order to prevent and reduce stress. The piglets were weaned at 18-21 days and the sows were moved to the mating unit in the same day. All in-all out system was used in mating, farrowing and nursery unit.

Selected piglets were identified individually by tattoo at day 7. After weaning, piglets were transported to 2 fattening farms, which was about 70 kilometers from breeder farm. Both fattening farms were evaporation houses which applied an all-in-all-out production system.

3.2.3 Environmental sample selection

There were three types of environmental samples, namely feed, water and house floor (table 7).

Feed: taken from the feed tray or the feeding box by using glove and placing it in a plastic bag

Water: taken from the drinking water by using glove and filling it into a sterile bottle

Housing swab: taken from different pens in the pig houses.

Table 7: Type and number of selected samples

Stages	Number	Sample types			Environmental samples			Total samples
		Fecal swab	Skin swab	25 g feces	Feed	Water	Pen swab	
day7	40 piglets	40	40	0	2	2	2	86
day18	40 piglets	40	40	0	2	2	2	86
day60	44 piglets	44	40	0	2	2	2	90
day90	44 piglets	44	44	36	2	2	2	130
day120	43 piglets	43	43	29	2	2	2	121
							Total	513

3.3 Collection of Samples

3.3.1 Faecal Samples

Faecal samples were meant for determining the current infection level in the respective pig herds. Individual faeces sampling (25-30 g) from a particular number of piglets at farm. Samples were taken by hand from the rectum, using a new disposal plastic glove.

The faecal samples will be submitted to the laboratory for examination within 3-4 hours of collection and cultural examination was undertaken on the same day of receipt.

If faecal samples needed to be stored, they were kept at 4 ^{o}C and tested within 24 hours.

3.3.2 Skin swab samples

At the day of collecting faecal samples, the skin swabs were tested for *Salmonella* simultaneously. Sterile cotton swabs were used. Each cotton swab is approximately 10 x 10 centimeter long (100 square centimeters) in the area of belly. Date, pen/house and farm were labelled then kept at 4 ^{o}C and tested with in 24 hours.

3.3.3 Pen Swab Samples

At the day of collecting faecal samples, the pens were tested for *Salmonella* presentation simultaneously. Sterile pairs of gauze socks were used (The pair of socks are consisted of an elastic cotton tube pulled over the investigator's boots, each sock is approximately 20 centimeter long). While walking through the pen, the cotton tubes absorbed faecal material. The investigator turned the socks inside out while walking so that all parts were exposed. One pair of socks was used for walking around the entire pen (approximately 30 steps).

After use, a soiled pair of sock was placed in a sterile plastic bag. Date, pen/house and farm were noted on the bag which was then kept at 4 ^{o}C and tested with in 24 hours.

This technique has been used to evaluate bacterial (*Salmonella*) contamination in the chicken house (Skov *et al*., 1999) and fattening pig house (Beloeil *et al*., 2004).

3.3.4 Water Samples

At least 1,000 ml of water were collected per sample by using a glove and filling the content into a sterile bottle. After collection, the sample was kept at 4 ^{o}C and sent to the laboratory for testing within 24 hours.

3.3.5 Feed Samples

25-30 g of feed was taken from feed tray or feeding box by using glove and keeping in plastic bag.

3.3.6 Overall numbers of samples

Overall, 513 samples were taken for *Salmonella* investigation, i.e. 276 fecal samples, 207 skin swab samples and 30 environmental samples.

3.4 Laboratory Procedure

3.4.1 Conventional Culture Method: ISO 6579 (2002)

- **Sample preparation**

25 grams of feces were mixed with 225 ml. of Buffered Peptone Water (BPW; Merck, Germany). The skin swab sample, faecal swab sample and floor swab sample were each put into 10 ml. of Buffered Peptone Water. 100 ml of water sample was mixed with 100 ml. of double-strength concentration of BPW. 25 grams of feed were mixed with 225 ml. of Buffered Peptone Water.

The conventional culture methods used were slightly modified from ISO 6579, which generally have four distinct phases or steps (Figure 3).

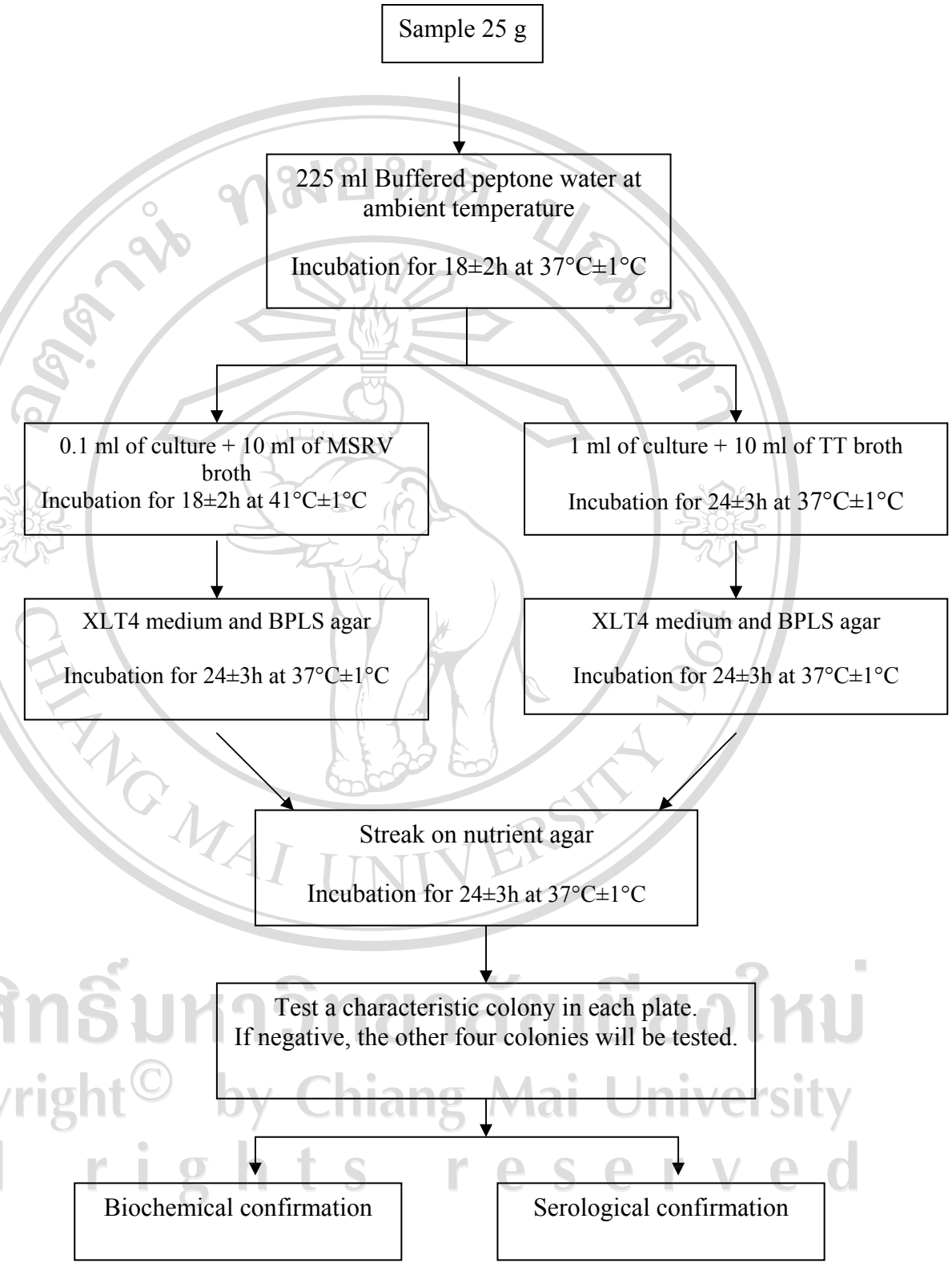


Figure 3; Conventional culture methods flow chart of *Salmonella*

1 Non-selective pre-enrichment: Samples were blended in a non-selective medium (Buffered Peptone Water) and incubated at 37 °C for 18-24 hours for allowing the resuscitation of any stressed organism as well as growth of all organisms as well.

2 Selective enrichment steps: During the reduction in numbers of accompanying organisms in the broth, the selective enrichment step supported the growth of the organism under investigation. Tetrathionate broth (TT) and Modified Semisolid Rappaport-Vassiliadis medium (MSRV) were used in this study. Incubation at 37°C±1°C and 41°C±1°C, for 24±3 hours and 18±2h for TT and MSRV, respectively.

3 Isolation step: Selective enrichment media were streaked on selective solid agars containing one or more agents that inhibit non-salmonella organisms. There were 2 selective solid agars used in this study, Brilliant-Phenol Red-Bile-Lactose-Saccharose Agar (BPLS) and Xylose lysine tergitol 4 agars (XLT4).

XLT4 is a highly selective plating medium used for isolation of *Salmonellae* from food, environmental and clinical samples. *Salmonella* colonies were black centered red colonies with a H_2S producer or red colonies with a non-producer.

On BPLS agar, the colony appearance of Salmonella was pink colonies surrounded by red zone.

4 Confirmation step: Characteristic colonies on the plates were submitted for biochemical testing in order to confirm whether the isolates were members of the

species *S. enterica*. Biochemical properties of Salmonella are shown in Table 4. Completing all the steps involved in this method requires at least 4 to 7 days, in order obtaining a definite diagnosis.

Table 8: Typical growth of *Salmonella* colonies on selective and differential media

Media	Colony appearance
BPLS	Pink colonies surrounded by red zone
XLT4	Black centered red colonies with H_2S producer, red colonies with non-producer

Table 9: Biochemical testing results of *Salmonella* (From: Institute of Meat Hygiene and Technology, Faculty of Veterinary Medicine, FU Berlin, Germany; Holt *et al.*, 2000)

Biochemical test	Bergy's Manual Result	Official collection Result
TSI glucose	+ (> 90%)	+ (100 %)
TSI gas	+ (> 90%)	+ (91.9 %)
TSI lactose	- (> 90%)	- (99,2 %)
TSI H_2S	+ (> 90%)	+ (91,6 %)
Urease	- (> 90%)	- (100 %)
LDC	+ (> 90%)	+ (94,6 %)
VPR	- (> 90%)	- (100 %)
Indole	- (> 90%)	- (98,9 %)

Biochemical confirmation

- **TSI agar**

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

Butt	
Yellow	glucose positive (glucose decomposed)
Red (unchanged)	glucose negative (glucose not decomposed)
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). When lactose-positive *Salmonella* is isolated, the TSI slant is yellow.

- **Urea agar**

Streak the agar slant surface. Incubate at 37 °C±1 °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. The reaction is often apparent after 2 h to 4 h.

- **L-Lysine decarboxylation medium**

Inoculate just below the surface of the liquid medium. Incubate at 37 °C±1 °C for 24 h ± 3 h. Turbidity and a purple colour after incubation indicated a positive reaction. A yellow colour indicated a negative reaction.

- **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 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.

- **Medium for Voges-Proskaur (VP) reaction**

Suspend a loopful of the suspected colony in a sterile tube containing 3 ml of the VP medium. 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.

- **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.

3.4.2 Serotyping

All isolates will be serotyped by agglutination according to the Kauffmann-White scheme using *Salmonella* Polyvalent I (A-E) and *Salmonella* Polyvalent II (J-67). Aaccording to the product from Sifin (Germany) and *Salmonella* antiserum specific to individual group by the following process (Figure 4).

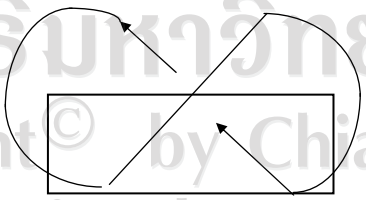
3.3.1 Test the selected colonies with *Salmonella* polyvalent I (A-E), if the agglutination is positive (+), the selected colonies posses the antigen to that antiserum, colonies will be regarded as a member of *Salmonella* Group A-E.

3.2.2 Test negative (-) result colonies (from the first step) with *Salmonella* polyvalent II (F-67), if the agglutination is positive (+), those colonies posses the antigen to that antiserum, the colonies will be regarded as a member of *Salmonella* Group F-67.

3.2.3 Serotyping of Somatic (O) antigens for *Salmonella* main groups determination (A (O 2), B (O 4,5,27), C (O 6,7,8,20), D (O 9,27,46,Vi), E (O 3,10,15,19,34)) by using a sequence of somatic antigen sera (Procedure based on manufacturer Safin, Germany).

3.2.4 After transferring sample isolation to the motility agar, flagella antigen determination was done. The agglutination of flagella antigen phase 1 and phase 2 was performed. In case of only 1 phase a challenge test, which the antigens were blocked by the particular H antiserum enforce the strain to develop the other phase was performed.

The object would be moved by slight rotation as shown in a picture.

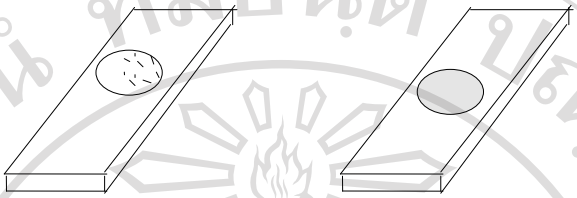


Slide moving direction

For assessment: Hold the object slide against a dark pad or a mirror.

Positive: Macroscopically detectable white agglutinated particles in the drop

Negative: homogeneous, cloudy liquid

Positive reaction  Negative reaction

For negative control:

The test must firstly be performed with physiological NaCl-solution and material of the suspicious colony; in case of agglutination: the strain can not be categorized.

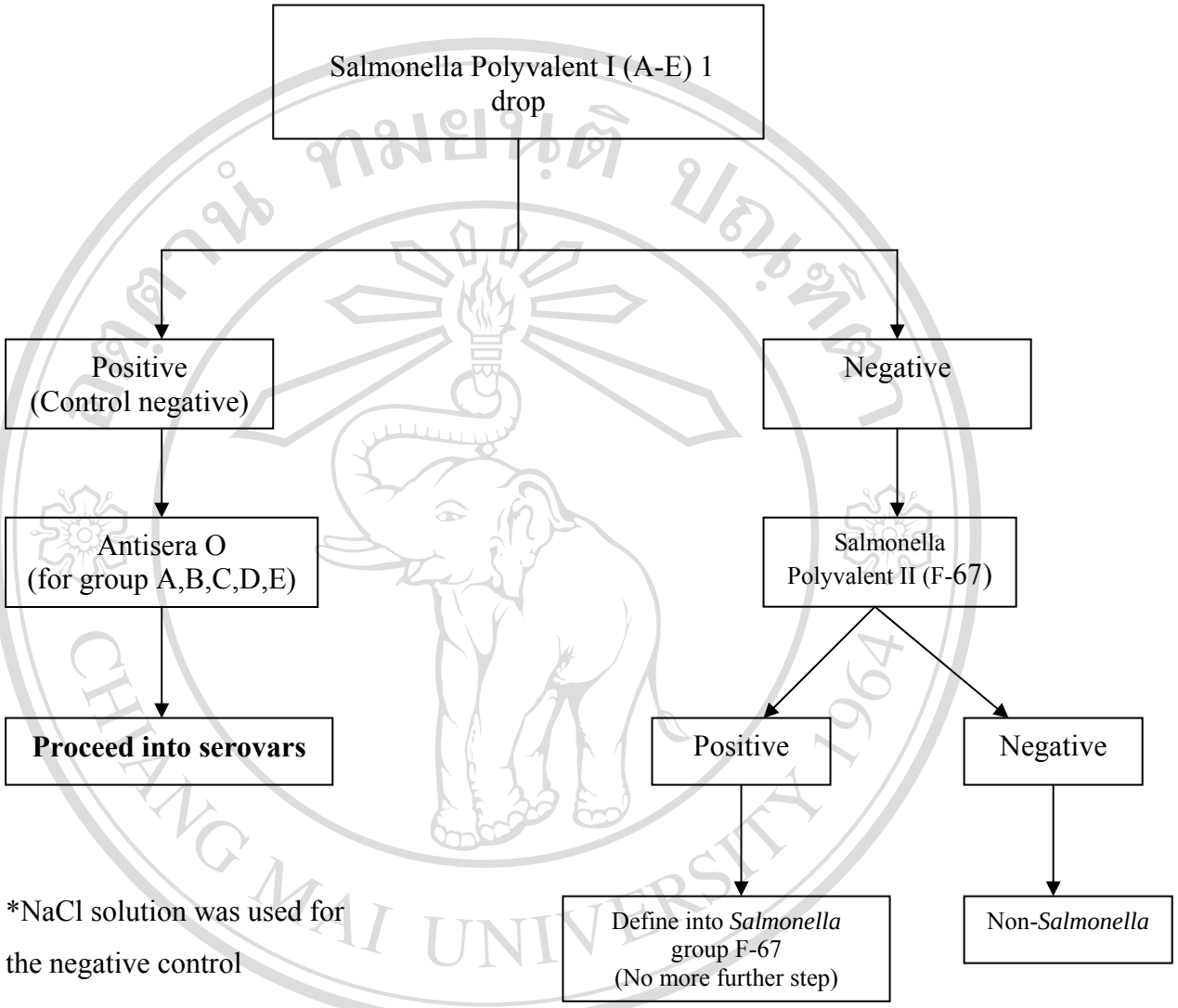


Figure 4; *Salmonella* serotyping flow chat (Polyvalent agglutination test)

3.5 Statistical Analysis

For descriptive analysis, Data from conventional *Salmonella* culture were entered into a STATA and were analyzed for

1. Salmonella prevalence in piglets and 95% confidence intervals (CI 95%) (separated by housing system (open and close) only at day 7 and 18).
2. Distribution of *Salmonella* serotypes in various types of samples.
3. Incidence of Salmonella in piglets at day 18, 60, 90 and 120 were calculated by this equation

$$\text{Incidence rate} = \frac{\text{Number of individuals that become disease during a particular period}}{\text{Average number of animal at risk during particular period}}$$

In the case of environmental sample, at least one sample was found positive; the piglet would be classified as *Salmonella* contamination (Beloeil *et al.*, 2004).