CHAPTER 3
MATERIALS AND METHODS

3.1 Study design

A cross-sectional study design was carried out during November 2008 to May 2009. 71 of the total of 87 broiler flocks from 77 contract farms of the study industrial poultry production company in Chiang Mai and Lamphun provinces were sampled. Sampling units were flocks, a flock being defined as "all poultry of the same health status kept on the same premises or in the same enclosure and constituting a single epidemiological unit. In the case of housed poultry this includes all birds sharing the same airspace" (EU Regulation 2160/2003/EC).

The flocks were sampled when chicks were one day old or within 3 weeks (EU Regulation 2160/2003/EC) before leaving for slaughter. If at least one of three samples was positive in the one-day-old chicks, the flock was classified as positive. If all samples of one-day-old chicks were negative, the flocks were re-sampled within 3 weeks before leaving for slaughter. Laboratory investigations and data analysis were carried out at Chiang Mai University.

3.2 Sample size and sample selection

3.2.1 Broiler flock selection

1. For broiler flock sample selection, a simple random sampling technique was used. 71 of the total of 87 flocks were selected by the simple random sampling technique.
2. Calculation of total sample size was based on an assumed flock prevalence of *Salmonella* of 50%, a 95% level of confidence and an accepted error of 5%. Using WinEpiscope® 2.0, the total sample size of 71 flocks was established.

3.3 Sample collection

Faecal samples

For collecting faecal samples from one-day-old chicks, it was not possible to use boot swabs/ socks to collect floor samples. In this case, faecal samples from the box liners were collected. To achieve 5000 chicks which represent 1 flock , 75 gram faeces per flock from 63 chick box liners (1 box contains about 80 chicks) were collected; the amount of faeces per one chick box was around 1.2 grams, then all individual faeces were pooled and divided into 3 samples of each 25 grams. If at least one of the 3 samples at stage one-day-old chicks was positive, the flock was declared as positive.

For collecting faecal samples from age of flock at within three week before leaving for slaughter, Samples were collected from each sample flock. According to EU Regulation 646/2007/EC, at least two pairs of boots/sock swabs were taken. In this study, three pairs of 'boot' or 'sock' swabs were taken from each sample flock. Each pair cover about 30% of the area of a flock house.

1. Boot swabs are a commercial absorptive paper/fabric overboot. Sock swabs comprise a 20 cm length of Tubegrip bandage which is bunched up around the ball of the foot. Sock swabs were turned by 90˚ to expose a clean surface three times during the sampling run but boot swabs were not turned.
2. To ensure that there can be no contamination of swabs prior to use or no contact with disinfectants, for example, plastic over boots were put on after walking through disinfectant boot-dips and not before.
3. A pair of new plastic over boots was put on in a house to be sampled before putting on the boot/sock swabs. It is normally easier to put on subsequent pairs of over- boots on top of a previous pair. One has to ensure that a new pair of plastic over- boots is used for each sample and that there can be no cross-contamination of boot swabs via hands when changing overboots or boot swabs. It is best to also use new disposable gloves for each change of overboots and boot swabs.

4. Before putting on the boot/sock swabs: the surface of the boot/sock-swabs or socks is moistened with maximum recovery dilutent (MRD: 0.8% sodium chloride, 0.1% peptone in sterile deionised water), or sterile water or any other dilutent approved by the National Reference Laboratory.
5. The floor area of a house was divided into three equal sectors for sampling to ensure that all sections were represented in the sampling in a proportionate way.
6. The investigator moved within a chosen sector so that at least 100 steps per pair of boot/sock swabs were covered, ensuring that all parts of the sector were sampled, including littered and non-littered areas but not including any outdoor areas in free-range flocks.
7. On completion of sampling in the chosen sector the boot/sock swabs were carefully removed so as not to dislodge adherent material. They were placed in a bag or pot and were labelled.

3.4 Laboratory Procedures

3.4.1. Conventional Culture Method: ISO 6579:2002, Annex D

Sample preparation

Each sample was mixed with 225 ml of Buffered Peptone Water (BPW; Merck, Germany). The culture methods used was carried out according to Amendment 1, Annex D of ISO 6579(2002) ("Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage"); the methods generally have four distinct steps (Figure 1)

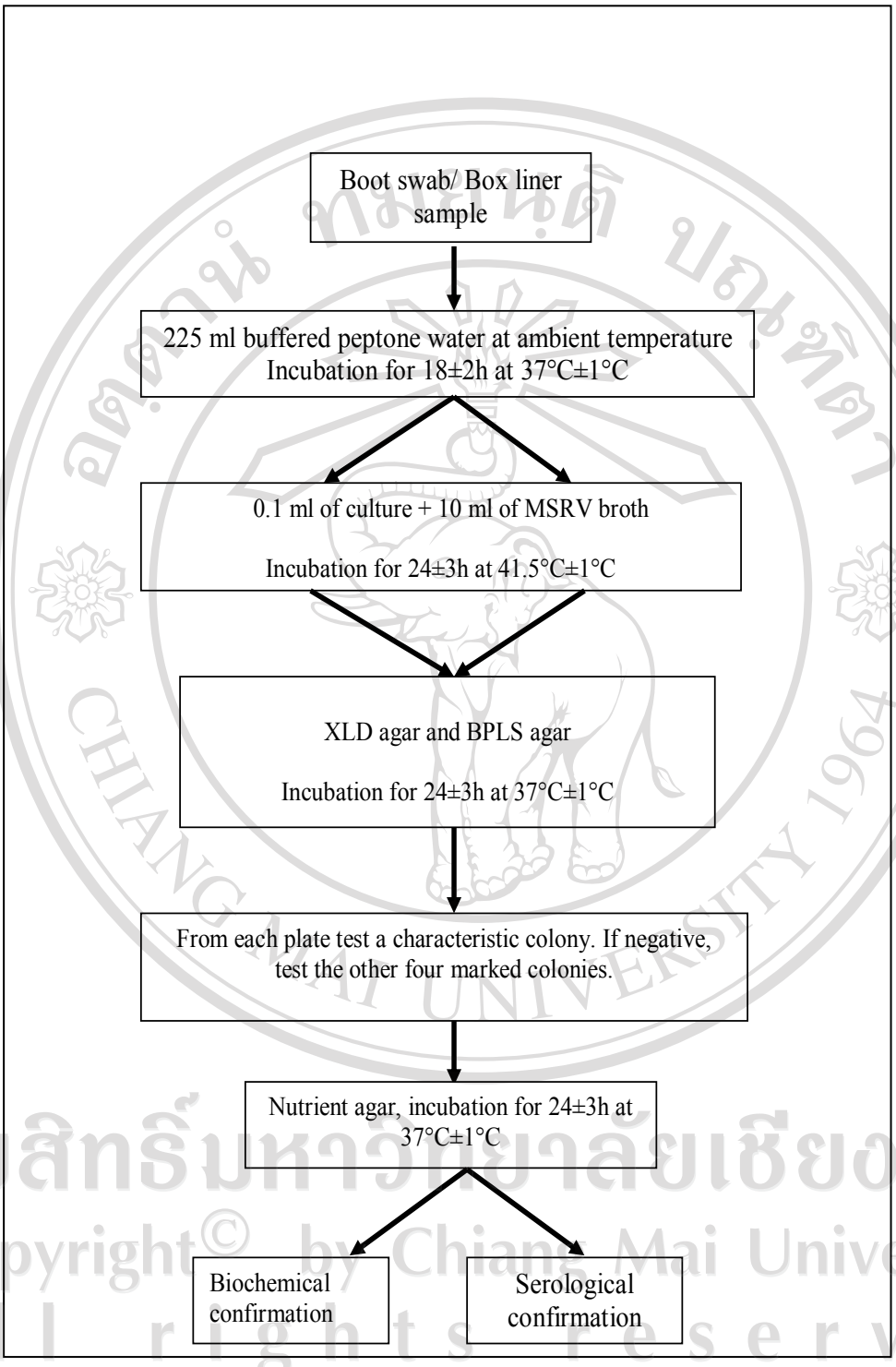


Figure 1: Conventional culture methods flow chart for *Salmonella* diagnosis

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 to allow the resuscitation of any stressed organisms as well as growth of all organisms.

2. Selective enrichment steps: This step allows the growth of *Salmonella* while suppressing competing microorganisms. Modified Semisolid Rappaport-Vassiliadis medium (MSRV) was used in this study. Incubation was at 41.5°C±1°C for 24±3 hours.
3. Isolation step: Selective enrichment media were streaked on selective solid agars containing one or more agents that inhibit non-*Salmonella* organisms. Brilliant-Phenol Red-Bile-Lactose-Saccharose Agar (BPLS) and Xylose Lysine Deoxycholate (XLD) agar were used in this study.
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*.

3.4.2 Serotyping

All isolates were serotyped by agglutination according to the Kauffmann-White scheme using *Salmonella* Polyvalent I (A-E) and *Salmonella* Polyvalent II (J-67) antisera (Manufacturer: Sifin, Germany) and *Salmonella* antisera specific to individual groups by the following process (Figure 2):

3.4.2.1 Selected colonies were tested with *Salmonella* polyvalent I (A-E); if the agglutination was positive (+), the selected colonies possessed the antigen to that antiserum and colonies were regarded as a member of *Salmonella* Group A-E.

3.4.2.2 Test negative colonies from the first step were tested with *Salmonella* polyvalent II (F-67); if the agglutination was positive (+), those colonies possessed the antigen to that antiserum and the colonies were regarded as a member of *Salmonella* Group F-67.

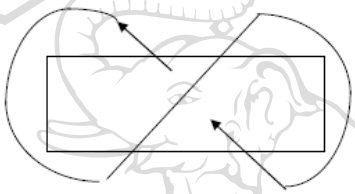
3.4.2.3 Serotyping of somatic (O) antigens for *Salmonella* determination of main groups (A (O 2), B (O 4,5,27), C (O 6,7,8,20), D (O 9,27,46,Vi), E (O 3,10,15,19,34)) by using a sequence of somatic antigen sera (Procedure based on manufacturer Sifin, Germany).

3.4.2.4 After transferring isolated samples 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, in which the antigens are blocked by the particular H antiserum to enforce the strain to develop the other phase, was performed.

Suspicious colonies were tested serologically using polyvalent sera I/II.

Technique:

A drop of the serum was put on a glass slide and rubbed in a suspicious pure colony. The slide was moved by slight rotation as shown in the picture:



For assessment:

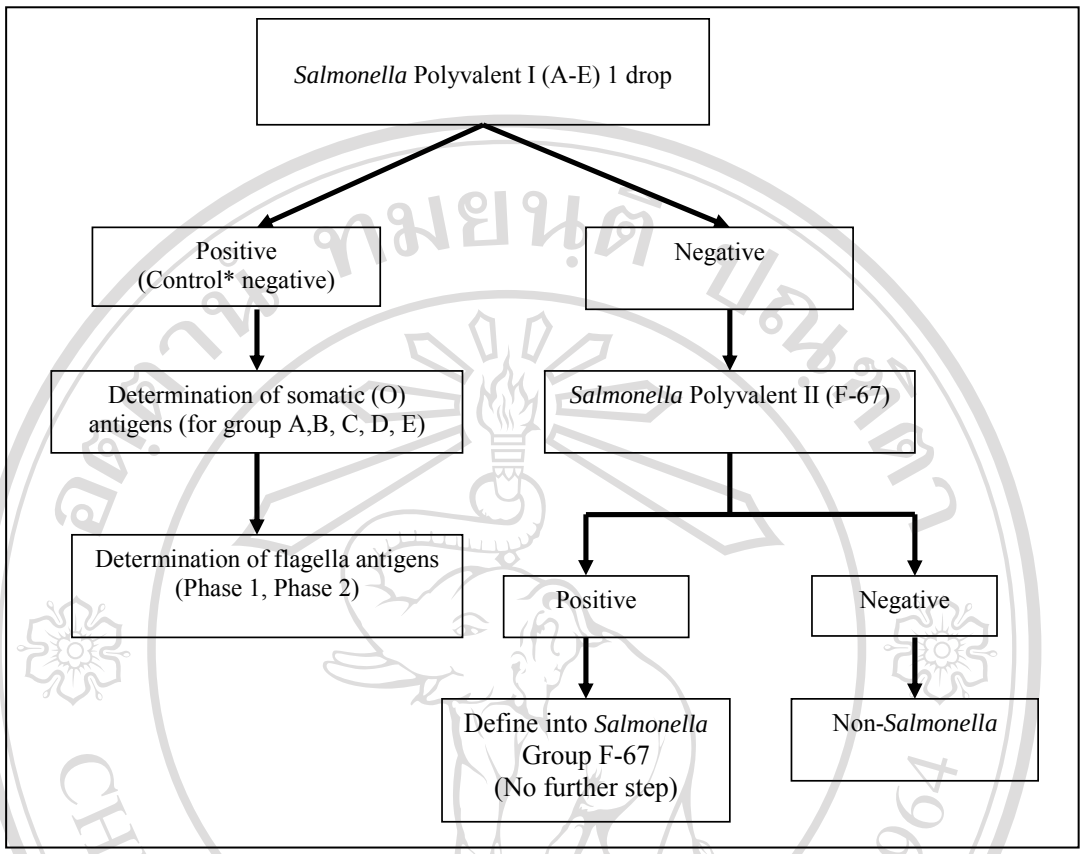
Positive: Macroscopically detectable white agglutinated particles in the drop

Negative: Homogeneous, cloudy liquid

Positive reaction Negative reaction

Check for self-agglutination:

The same analysis was carried out with physiological NaCl-solution and material of the suspicious colony; in case of agglutination, the strain was untypable.



(Source: Institute of Meat Hygiene and Technology, Faculty of Veterinary Medicine,FU Berlin, Germany, Vers.1 (June 2004)

Figure 2: *Salmonella* serotyping flow chart (Polyvalent agglutination test)

3.5 Questionnaire for potential risk factors associated with *Salmonella* flock prevalence

Data per flock concerning potential explanatory factors for flock prevalence followed the EU format (EFSA, 2007b) and included flock characteristics, holding characteristics and management/production parameters. Questionnaires were administered to each farmer.

Questionnaire data collection

1. Age of broilers at sampling (in days)
2. Strain reared in flock.

a) Arber Acre b) Ross c) others

3. Number of the birds raised per year in the holding ……birds
4. Number of birds in the poultry house……birds
5. Number of crops (flock cycle) per year
6. Number of flocks in the holding at time of sampling……..
7. Number of broilers in flock tested
8. Number of years since the construction or last reconstruction of poultry house

a) <5 years b) ≥ 5

9. Visual detection of beetles/flies/other insects by interviewer during rearing

a) Yes b) No

10. Are there any animal species other than poultry in farm?

a) Yes b) No

10.1 If Yes, which kinds of animals……Number…….

11. Numbers and types of persons (producer, including farmer, worker and visitor) entering the poultry house during rearing.(at the time before interview and collecting sample)

a) ≤ 2 producers or workers, no visitor b) ≤ 2 producers or workers, with visitor

c) >2 producers or workers ± visitors

12. Source of feed
13. Texture of feed

a) Pellet b) Meal

14. Kind of water source used in farm

a) Tap water b) Deep well

c) Surface well d) More than one source

15. Is there any treatment of drinking water for birds before use?

a) Yes b) No

16. Have salmonella vaccinations been used during rearing?

a) Yes b) No

17. Have antimicrobials has been used during the last two weeks prior to sampling?

a) Yes, the name of antibiotic…… b) No

18. Information on disease history

a) Treatment history

b) Which kind of antibiotic used?

c) Days in use

d) Duration of treatment

19. Assessment of hygiene practices in farm

a) Poor b) Moderate c) Good

3.6 Statistical analysis

A flock was considered positive if the presence of *Salmonella* spp. was detected in at least one of the three samples.

Data were entered into an Excel spreadsheet. Analysis and reporting of data followed the EU format (EFSA 2007a, b): Data validation and cleaning followed the data exclusion criteria of Annex I of EFSA (2007a). Only the observed prevalence was investigated. In statistical terms, this translates into the assumption that no false-negative flock occurred in the survey. Investigation of the effect of potential risk factors which may be associated with the occurrence of *Salmonella* followed Annex I of EFSA (2007b) and included correlation analysis.