3. MATERIAL AND METHODS

3.1. Study Design

A cross-sectional study design was used. Samples were collected and questionnaires were administered to each farm during December 2004 to May 2005.

3.2. Sample Size and Sample Selection

3.2.1. Sample Size Determination

In order to estimate the prevalence of *Salmonella* infection in pre-slaughter pigs in the Chiang Mai province, using the prevalence of 69.5% (Patchanee *et al.*, 2002) on a pig level with a maximum allowable error of 8% and 95% confidence level, 420 fattening pigs (about 1-3 days before slaughter, 90-100 kg live weight) were selected conveniently for individual blood sampling and 194 pigs were selected for faecal sampling (Daniel, 1987). Questionnaires were used to collect the management information of those herds. Environmental samples related to the risk of introducing *Salmonella* into the herd, including house floor and water supply, were collected and tested for the presence of *Salmonella.* A convenient sample of 22 pig herds was observed in this study.

3.2.2. Farm and Pig Selection

A total of 22 farms was selected from 2 groups. The first group had open house (17 farms), the second group was environment-controlled farms (5 farms). For each farm, twenty pigs were selected for blood sampling, and 10 of these 20 pigs were selected for faecal sampling.

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3.2.3. Environmental Sample Selection

Two types of environmental samples, a water sample and a floor swab sample were collected. Water samples included (i) water used for cleaning and disinfection, (ii) drinking water and (iii) waste water. Seven pens in each farm were selected for floor swabbing.

3.3. Collection of Samples

3.3.1. Serum Samples

Blood samples, each 10 milliliter, were taken at slaughter during bleeding and collected in test tubes individually. Each tube was labeled with each pig's unique identification number and centrifuged to separate serums and platelets. Then, the serum was removed from each blood sample and stored at -20 ^{o}C until tested.

3.3.2. Faecal Samples

Faecal samples were used to indicate the current infected proportion in the respective pig herds. Individual faecal samples (25-30 g) were collected by hand per rectum, using new disposal gloves. The faecal samples were submitted to the laboratory for examination within 4 hours after collection and processed on the same day of collection or kept at 4 ^{o}C and processed within 24 hours.

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3.3.3. Pen Swab Samples

Pen swabs were collected on the same day as faecal samples and tested for *Salmonella* presentation simultaneously. A sterile pair of gauze socks was used. The pair of socks consisted of an elastic cotton tube, each sock was sized approximately 15x20 centimeters. The socks were pulled over the investigator's boots. The investigator walked through the entire pen (approximately 30 steps) and turned the

socks during sampling to allow all parts of the socks to be exposed and to absorb faecal material. A soiled pair of socks was placed in a sterile plastic bag with 225 ml of peptone water. The labeled bags were kept in an icebox and put in the incubator at 37 ^{o}C within 3-5 hours after collection. This sampling technique has been used to evaluate bacterial (*Salmonella*) contamination in the chicken house (Skov *et al*., 1999) and the fattening pig house (Beloeil *et al*., 2004).

3.3.4. Water Samples

Each water sample comprised 1,000 ml in a sterile bottle. Samples were kept at 4 ^{o}C and sent to the laboratory for testing within 3-4 hours after collection.

3.4. Laboratory Procedures

3.4.1. Serology; ELISA

The commercial test kit SALMOTYPE® Pig LPS ELISA (Labor Diagnostik Leipzig, Germany) was used.

The kit is an enzyme immunoassay for the detection of antibodies specific to *Salmonella* in pork meat juice or pork serum, it detects antibodies to the O-antigens 1, 4, 5, 6, 7 and 12. The SALMOTYPE® Pig LPS ELISA detects more than 90% of the most common *Salmonella* serotypes in the Western European area.

This assay is designed to measure the quantity of antibodies to *Salmonella* in pork meat juice or in pig serum. The *Salmonella* antigen is coated on 96-well plates. Upon incubation of the test sample in the coated well, antibodies specific to *Salmonella* form a complex with the coated *Salmonella* antigen. Unbound material is washed away and a conjugate is added which binds to any bound pork antibody in the wells. After washing away unbound conjugate from the wells, enzyme substrate is added. Subsequent colour development from the conjugate-bound enzyme is directly

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related to the amount of antibodies to the *Salmonella* present in the test sample (Figure 2).

The ratio of the OD values of the controls and their concentrations give a linear regression line. The linear regression line is calculated by plotting the OD values of control on the X-axis versus the measured OD-values on the Y-axis. The antibody concentration of the samples has to be calculated by use of the straight-line formula.

- Cut-off values for samples (serum, meat juice, plasma):

  $\geq$ 40 OD% positive

  20 - < 40 OD% weak positive

  10 - < 20 OD% doubtful (positive)

  < 10 OD% negative
- Cut-off values of samples for categorization of stocks according to monitoring programs:

  $\geq$ 40 OD% or $\geq$ 20 OD% are positive depending on national regulations

For the assay to be valid, the P/N-quotient between the Positive Control Serum 1 (P) and the Negative Control Serum (N) should be greater than 4.0.

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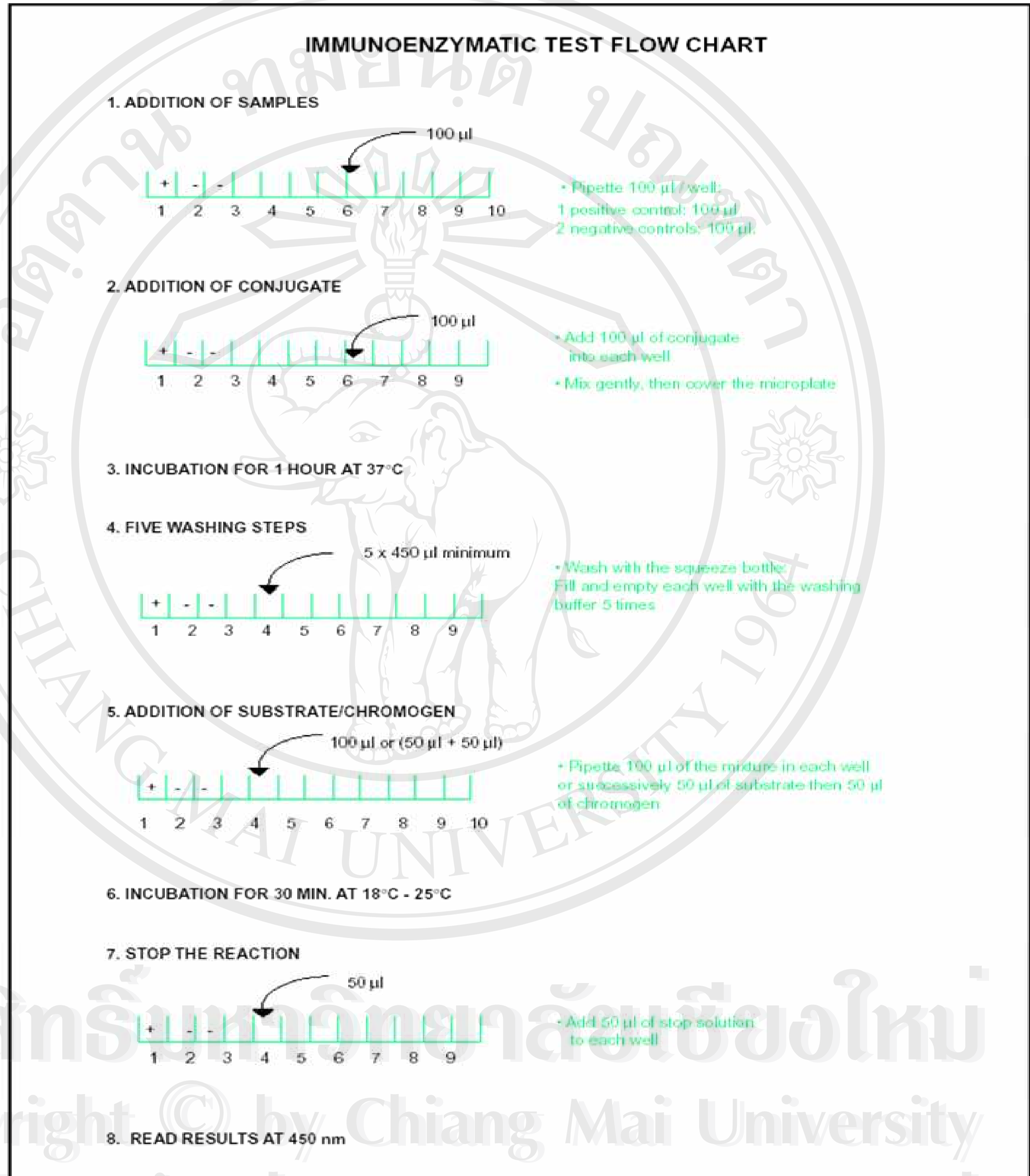
Figure 2: ELISA test flow chart



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Source: Axelsson and Sorin (1997)

3.4.2. Conventional Culture Method

The conventional culture methods used were slightly modified from ISO 6579 (2002); Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. The protocol generally has four distinct phases or steps (Figure 3).

Step 1. Non-selective pre-enrichment: The sample was blended in a nonselective medium and incubated at 37 °C for 18-24 hours to allow resuscitation of any stressed organism and growth of all organisms as well.

Step 2. Selective enrichment step: To allow growth of the organism under investigation, while reducing the numbers of accompanying organisms in the broth. Two types of selective enrichment media were used in this study. The first media used was Tetrathionate broth (Merck® Ltd.), another media used was the Rappaport-Vassiliadis medium (Merck® Ltd.).

Step 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, the first one was BPLS (Brilliant-Phenolred-bile-Lactose-Saccharose Agar, Merck® Ltd.) and the second one was XLT4 (Xylose lysine tergitol 4 agar**,** Merck® Ltd). XLT4 is a highly selective plating medium used for the isolation of salmonellae from food, environmental and clinical samples. The properties of *Salmonella* colonies are described in Table 5.

Step 4. Confirmation step: Characteristic colonies on the plates were submitted for biochemical testing and seroagglutination testing to confirm that the isolates were members of the species *S. enterica*. Biochemical properties of *Salmonella* are shown in Table 6.

Completing all the steps involved in this method required at least 4-7 days, in order to obtain a definite diagnosis of *Salmonella*.

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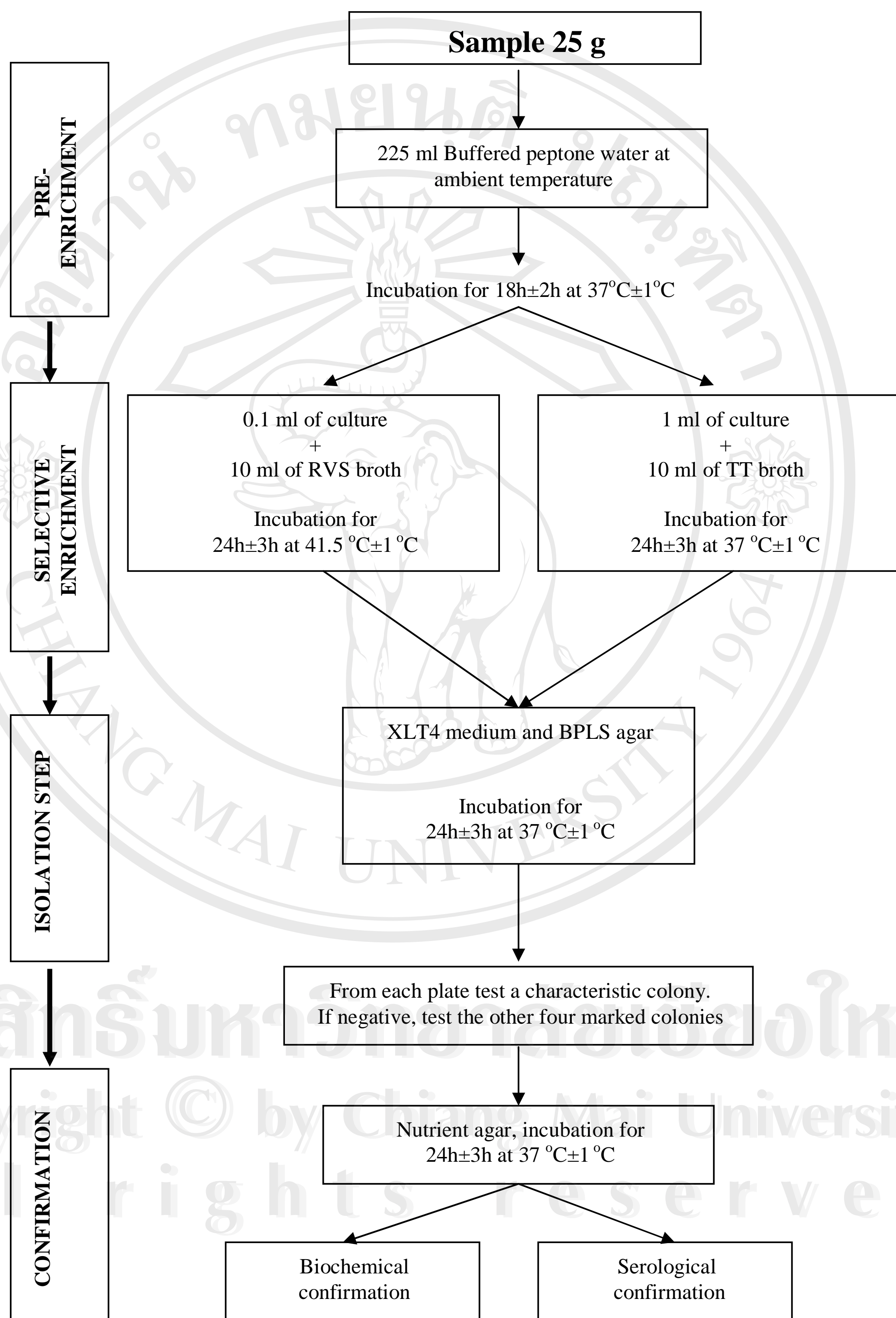
Figure 3: Flow chart of *Salmonella* conventional culture methods



Source: Adapted from ISO 6579 (2002)

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Table 6: 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 7: Biochemical testing results of *Salmonella*

Biochemical test	Bergy's Manual Result	Official collection Result
Glucose from TSI	+ (> 90%)	+ (100 %)
Gas from TSI	+ (> 90%)	+ (91.9 %)
Lactose from TSI	- (> 90%)	- (99.2 %)
H_2S from TSI	+ (> 90%)	+ (91.6 %)
Urease	- (> 90%)	- (100 %)
Lysine decarboxylation	+ (> 90%)	+ (94.6 %)
Voges-Proskauer reaction	- (> 90%)	- (100 %)
Indole	- (> 90%)	- (98.9 %)

Source: Holt *et al.*, 2000, Institute of Meat Hygiene and Technology, Faculty of Veterinary Medicine, FU Berlin, Germany)

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3.4.3. Serotyping

All isolates were serotyped by agglutination according to the Kauffmann-White scheme using *Salmonella* Polyvalent I (A-E) and *Salmonella* Polyvalent II (F-67) (Sifin, Germany) and *Salmonella* antiserum specific to the individual group by the following process (Figure 4).

1. Test the selected colonies with *Salmonella* polyvalent I (A-E), if the result was positive (+), the selected colonies possessed the antigen to this group, colonies were regarded as a member of *Salmonella* group A-E.

2. Test negative (-) result colonies (from the first step) with *Salmonella* polyvalent II (F-67), if the result was positive (+), those colonies possessed the antigen to this group; colonies were regarded as a member of *Salmonella* Group F-67.

3. Serotyping of Somatic (O) antigens to determination *Salmonella* 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). Sequence of testing based on information of the occurrence in Thailand and South East Asia.

4. Determination of flagella antigens, this step was done after transfer of the isolate to the motility agar. Performing agglutination for flagella antigen phase 1 and phase 2. If phase 2 did not appear, the serotype might be in the first phase only or vice versa. Then proceeding with the challenge test, where the antigens were to be blocked by the particular H antiserum to force the strain to develop the other phase (procedure based on manufacturer Sifin, Germany).

5. Diagnosis of the serotype of *Salmonella*.

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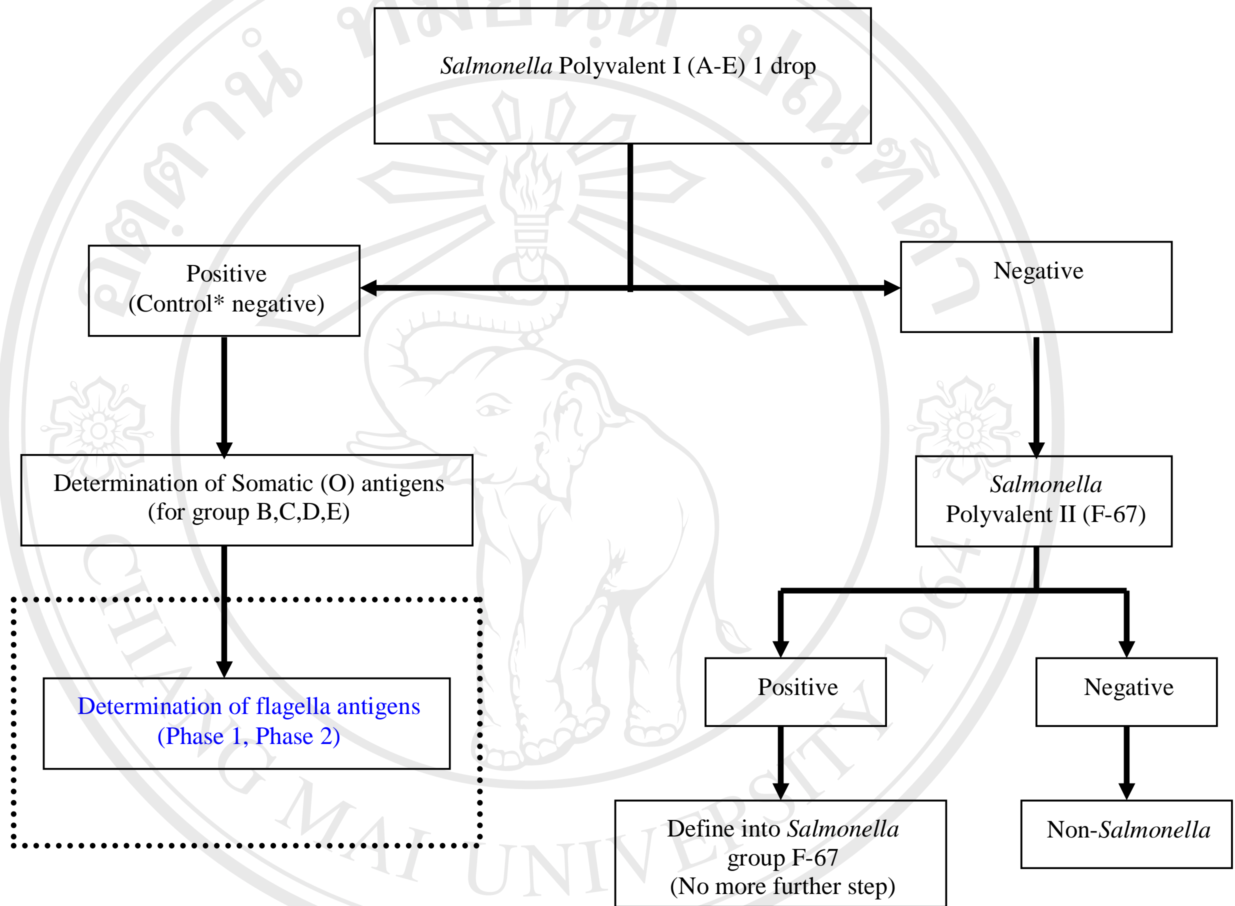
Figure 4 : *Salmonella* serotyping flow chat



* The negative control used was NaCl solution

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3.5. Questionnaires Survey

A specific questionnaire was administered to each farmer by the author. Data concerning the general characteristics of the farm and the premises, biosecurity procedures, type of feeding and the rearing characteristics of the batch during finishing periods were collected. In addition, the on-farm technical documents were examined for this purpose too.

The questionnaires and check lists were used for estimation of the management in each selected farm. Factors affecting the occurrence of *Salmonella* in fattening-pigs, and which were parts of the questionnaire, are shown in Table 8.

3.6. Statistical Analysis

For descriptive analysis, herds were considered seropositive when one or more blood sample was found positive. All herds, in which *Salmonella* was cultured from one or more samples, were considered bacteriological positive. The statistical analysis in use was

1. Chi square test for univariate risk factor analysis. This was to evaluate the impacts of each factor to the prevalence of *Salmonella* in faecal isolation and in the serological test
2. Logistic regression model for multivariable analysis. All relevant factors were included in the model. This was to evaluate the impacts of particular risk factors without interaction from the other factors (David, 1994).

The statistical programs used were EpiCalc 2000, NCSS 2000, Win Episcope 2.0, Intercooled Stata 6, Epi Info 2002, SAS statistic program.

In the case of environmental samples, if at least one sample was found positive, the herd was classified as *Salmonella* contaminated (Beloeil *et al.*, 2004).

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Table 8: Summary of questionnaires and checklist

Cluster	Factors
Animals	Kind of animals, number, origin and breed.
Integrated quality control program	Whether or not, and if so, which program.
Feed and feeding system	Which antibiotic growth promoter, type of feeding and type of drinking water and watering system. Feed storage and sanitation.
Housing	Number of house/pen, total number of compartments, number of animals per compartment, type of floor, type of slurry or waste management system.
Medication and vaccination	If, when, why and what sort of medication, dose rate and duration of treatment. Type of vaccine and probiotic used.
Hygiene	All-in/all-out procedure, cleaning and disinfection procedure, chemicals used, methods of fly and rodent control, personal hygiene and number of visits by vets, isolation of sick animals
Production parameters	Average daily gain (ADG), feed conversion ratio, mortality and the percentage of loss during fattening.

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