CHAPTER 3
MATERIALS AND METHODS

3.1 Study design

This study was a comparative microbiological survey based on samples collected in slaughterhouses.

3.2 Study location

The study was performed in ten licensed pig slaughterhouses in Hanoi from December 2008 to April 2009. The number of pigs slaughtered per day in each slaughterhouse varied from 15 to 60.

The slaughterhouses consisted of 4 modern slaughterhouses (code numbers 3, 7, 8, 9) and 6 traditional slaughterhouses (coded as 1, 2, 4, 5, 6, 10). All of them started working at 1 a.m to 4 a.m in the morning. However, in order to save expenditure on energy, maintenance and hygiene measures, 3 of the modern slaughterhouses (No. 7, 8, 9) used the floor-dressing method instead of hoisting line systems. Thus, only 1 slaughterhouse was classified as hoisting slaughterhouse and the other 9 as floor-dressing slaughterhouses.

Based on the characteristics of the eviscerating floor surface (smooth or rough), the scalding practice (dipping the carcass into a vat of hot water or pouring hot water on the carcass) and the pressure of the cleaning water (high pressure with pumping or lower), the 9 floor-dressing slaughterhouses were divided into 5 types (***Table 2***).

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Table 2 Some characteristics of floor-dressing slaughterhouses

	Evisceration floor characteristic	**Scalding method**	**Water pressure for C&S**
Type 1 (SLH 1)	Rough	Pouring hot water on the carcasses	Low
Type 2 (SLH 2)	Smooth	Pouring hot water on the carcasses	Low
Type 3 (SLH 4)	Smooth	Dipping carcasses into a vat	Low
Type 4 (SLH 7,8,9)	Smooth	Dipping carcasses into a vat	High
Type 5 (SLH 5,6,10)	Rough	Dipping carcasses into a vat	Low

(SLH: slaughterhouse)

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3.3 Sample sites

Table 3 Sample collecting sites

Area	Hoisting slaughterhouse	Floor-dressing slaughterhouse
Pig pen	Floor	Floor
Evisceration	Hooks Knife 1	Floor 1 Knife 1
Carcass splitting	Saw	Floor 2 Knife 3
Meat cutting, trimming	Knife 2	Cutting table Wooden board Knife 4 Hooks
Carcass weighing		Scale

The samples were collected in pig pens (lairage area), on the eviscerating floor (floor 1), on the carcass splitting floor (floor 2), at meat cutting or trimming and at carcass weighing areas. Swabs were taken from floors, knives for evisceration (knife 1), knife for trimming (knife 2), knives for carcass splitting (knife 3), knives for offal separating (knife 4), saw, hooks, wooden boards, meat cutting table and scales before and after cleaning and sanitation.

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3.4 Sampling scheme

During each time of a slaughterhouse investigation, five samples were collected at the described areas after the work shift had started, with 10 – 15 pigs slaughtered. These samples were representative of the situation before cleaning and sanitation. About 30 minutes after cleaning and sanitation, another five samples were taken at identical areas ***(Figure 4)***.

In fact, in the hoisting slaughterhouse, all five sampling sites indicated in Table 3 were sampled at each visit. However, in floor-dressing slaughterhouses, there were 10 sites chosen for sampling but only 5 to 6 sites, depended on individual slaughterhouse, were sampled in every visit.

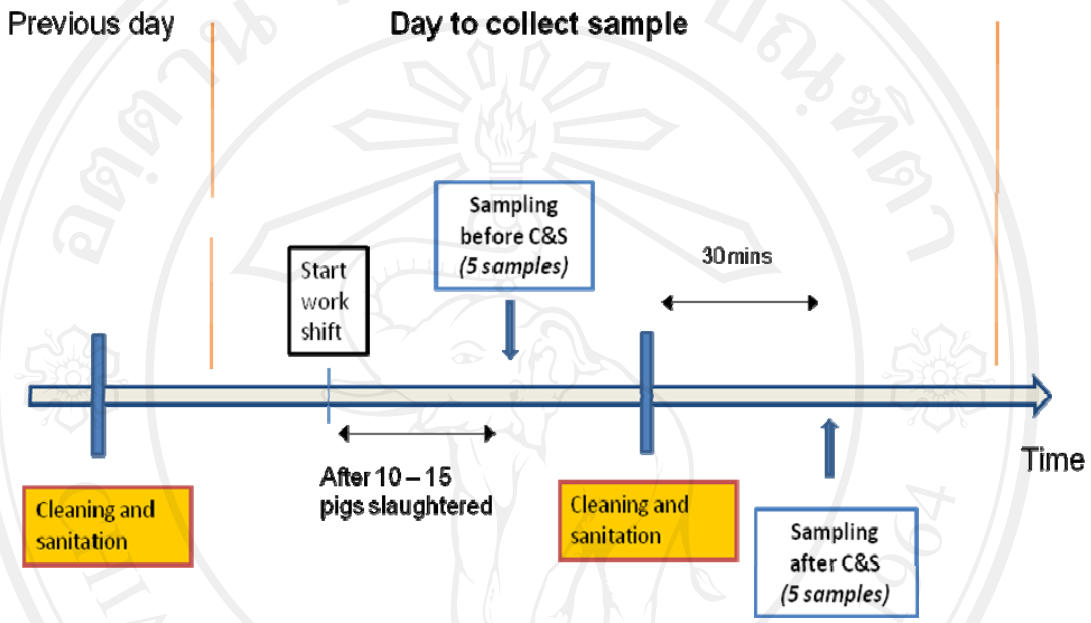

Figure 4 The sampling scheme for a one-day investigation

3.5 Frequency of sampling

Each slaughterhouse was investigated and samples collected five times consecutively. The resulting total number of samples was 500 as shown in Table 4.

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Table 4 Frequency of sample collection

Slaughterhouse	**Number of samples at one examination day**		**Frequency of visits**	**Total of samples taken**
	Before C&S	After C&S		
1	5	5	5	50
2	5	5	5	50
3	5	5	5	50
4	5	5	5	50
5	5	5	5	50
6	5	5	5	50
7	5	5	5	50
8	5	5	5	50
9	5	5	5	50
10	5	5	5	50
Total			50	**500**

3.6 Sampling method

The wet-dry double swab technique was applied.

For the floor and surface: a sterile cotton swab was soaked in a 10 ml tube of buffered peptone water (BPW) for moistening, then was streaked under pressure on the surface within the area of a template (10 x 10 cm^2) and transferred to a BPW tube. Next, a second dry cotton swab was streaked again on that surface and transferred to the same BPW tube. Enterobacteriaceae counts were calculated in 1 cm^2 of the surface.

For equipment such as knives, saw, hooks: the wet-dry double swab technique was done without use of the template on both sides of the knife edge, saw blade and hooks where the most contact sites to carcasses were located. In this case, 10 ml

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sample tube was considered as a constant factor with numbers of Enterobacteriaceae. Enterobacteriaceae counts were calculated in 1 ml from the sample tubes.

Samples were labeled and sent to the laboratory within the day of collection.

3.7 Laboratory procedures

The samples collected were analyzed in the microbiology laboratory of the National Center for Veterinary Hygiene No. 1 in Hanoi.

Media preparation followed the manufacturer's instructions shown in appendix 1.

Isolation and identification of Enterobacteriaceae followed ISO 21528-2:2004 ***(Figure 5)***.

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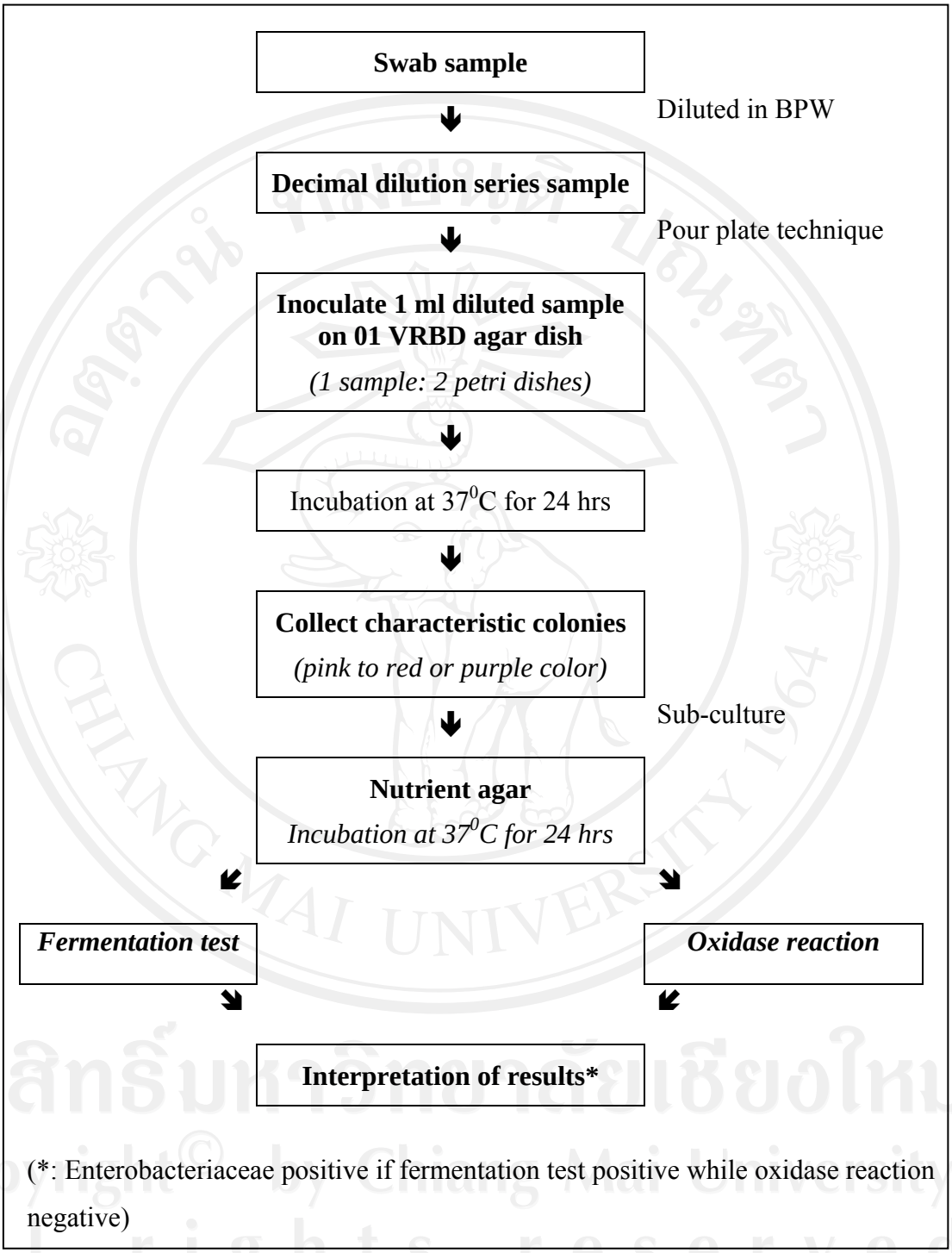

Figure 5 Enterobacteriaceae isolation and identification flow chart (ISO 21528-2:2004)

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3.7.1 Preparation of test sample dilutions

An initial sample tube was shaken by the vortex well for 10 seconds, then 1ml of the suspension was transferred into a new 9 ml BPW tube to get 10 ml of diluted sample at 10^{-1} dilution. Dilution was continued in the same manner for the next decimal dilutions. Finally, a decimal dilution series from the test sample was obtained.

3.7.2 Inoculation and incubation

- Two sterile petri dishes were taken for each dilution. The test sample tube was shaken well. A sterile pipette was used to transfer 1 ml of the test sample to each dish. The same pipette was used to transfer from the highest decimal dilution to the lowest dilution of the same series.
- Into each inoculated petri dish approximately 10 ml of the crystal-violet neutral-red bile glucose agar (VRBD agar) medium was poured at 45^0C. The inoculums were mixed gently and the medium was allowed to solidify.
- After the mixture had become completely solidified, a covering layer of approximately 15 ml of the VRBD medium was added. The dish was let to become completely solidified at room temperature.
- The prepared dishes were inverted and incubated in the incubator at 37^0C for 24 hrs ± 2 hrs.

3.7.3 Counting and selection of colonies for confirmation

Characteristic colonies were pink to red or purple, with or without precipitation haloes.

Dishes containing less than 150 characteristic colonies were selected and the colonies were counted. Then five colonies were randomly chosen for sub-culturing for biochemical confirmation tests.

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3.7.4 Sub-culturing of selected colonies

Each selected colony was streaked onto nutrient agar dishes and incubated at 37^0C for 24 hrs ± 2 hrs.

3.7.5 Biochemical confirmation tests

Well-isolated colonies from the nutrient agar were selected for the biochemical confirmation tests.

3.7.5.1 Oxidase reaction

A sterile loop was used to take a portion of each well-isolated colony, then streaked onto a filter paper moistened with the oxidase reagent. A result was negative when no dark blue color did appear within 10 seconds.

3.7.5.2 Fermentation test

Using a sterile wire, colonies that had given negative results to the oxidase test were selected and each colony was inoculated into 2 glucose agar tubes. One tube was covered with 1 ml of sterile liquid paraffin for anaerobic condition and the other without paraffin for aerobic condition. The result was positive when the medium changed its color from purple to yellow because of pH reduction.

3.7.6 Interpretation of the biochemical tests

Colonies with oxidase negative and fermentation positive results were confirmed as Enterobacteriaceae.

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3.7.7 Calculation of Enterobacteriaceae counts

In this study, swab samples were diluted in decimal dilution series, therefore, Enterobacteriaceae counts were calculated from surface (in $\log_{10}$ cfu/cm^2) and from equipment such as knives, hooks and a saw (in $\log_{10}$ cfu/ml) as following:

$$N1 = \frac{\Sigma C}{[(1 * n_1) + (0.1 * n_2)] * (d) * 10} \quad ; \quad N2 = \frac{\Sigma C}{[(1 * n_1) + (0.1 * n_2)] * (d)}$$

Where N1 = Number of colonies per cm^2

N2 = Number of colonies per ml

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 = Decimal dilution from which the first counts were obtained

Subsequently, N1 and N2 were converted into log 10

3.8 Data management and analysis

3.8.1 General information form of slaughterhouses

A general information form was used to collect background information on the pig slaughterhouses at the time of visit ***(Appendix 2)***.

3.8.2 Laboratory test result form

This form recorded sample analysis results ***(Appendix 3)***.

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3.8.3 Database and bio-statistical analysis

- A database with results from questionnaires and with slaughterhouse and laboratory data was set up in the Microsoft Office Excel program.
- Comparisons of Enterobacteriaceae counts before and after cleaning and sanitation were carried out by the Student's *t*-test.
- Comparisons of Enterobacteriaceae counts in more than 2 areas, on equipment or for slaughterhouse types were done by the ANOVA test.
- Software for bio-statistical analysis was Stata SE 9.2 and Endnote X1 was used to insert references.

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