3. MATERIALS AND METHODS

3.1 Study design

3.1.1 Study design

This is a cross-sectional observation study carried out using retail pork in the Hanoi area and questionnaire surveys on practices of meat selling and pig husbandry in the Hanoi suburban area and the neighboring provinces that supply pork to Hanoi market.

3.1.2 Study site

The study site is shown as in figure 1. It consists of:

There are fourteen districts, of which five are suburban districts (Thanh Tri, Gia Lam, Dong Anh, Soc Son and Tu Liem) and nine urban districts (Cau Giay, Long Bien, Hoang Mai, Tay Ho, Thanh Xuan, Hai Ba Trung, Ba Dinh, Hoan Kiem, and Dong Da). The study was conducted in all these districts by collecting retail fresh pork samples and obtaining data from the questionnaire surveys on selected factors relating to practices of meat selling and pig husbandry. According to the Hanoi Sub-Department of Animal Health (CCTYHN, 2005) the pork production in Hanoi met only two third of the total demand of the whole city in 2005. The rest of the pork demand was supplied by the neighboring provinces around Hanoi, mainly by Ha Tay, Ha Nam, Vinh Phuc and Hung Yen provinces. Thus, these provinces were also selected for conducting the questionnaire survey on selected factors relating to the pig husbandry practice.

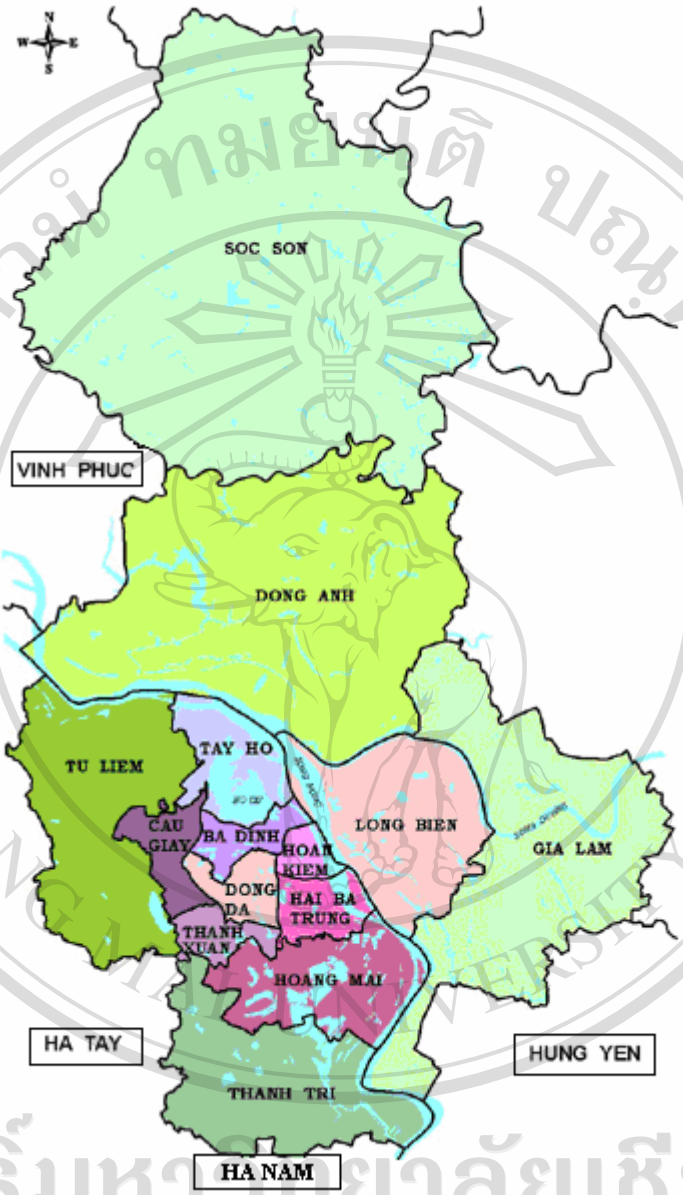

Figure 1: Map showing the sites selected for pork sampling and questionnaire surveys in Hanoi (coloured) and adjacent provinces

(Adapted from administrative borders of the municipality of Hanoi

3.1.3 Study population

Sampling sites were recruited as permanent meat shops in all districts of Hanoi but only a certain percentage of shops that were sufficient in terms of a minimum of statistically required analysis samples. The sampling unit was a pork shop which sold more than 50 kg/day. The population size (N) was 1200 shops (Duong, 2005). The analysis sample was a cut piece of retail fresh pork with approximately 100 to 120 g/sample.

3.1.4 Sample size

Since there are only some few reports on the prevalence of resistant *E. coli* in pork in Vietnam the reported prevalence of resistant *E. coli* in pork in studies of To (2004, 2006) was used for the sample size calculation. However, there was no data on the prevalence of pork *E. coli* resistant to oxytetracycline, cephalothin, trimethoprim and sulphonamides. So the hypothesis was that the expected antimicrobial resistance prevalence of *E. coli* to every antimicrobial concerned was 50 %. Using the win-Episcope 2.0 with population size (N) of 1200, with a 95 % confidence interval (CI) and 5 % of accepted error, the required sample size for determining the prevalence of antimicrobial resistance of *E. coli* isolated from retail fresh pork in Hanoi market, Vietnam, was 292. Since the prevalence of *E. coli* contaminated in pork marketed in Hanoi, is 76.7 % (Do *et al.,* 2006), with 5 % of the number for any loss to follow up study, the final required minimum number of samples (n) is:

$$n = \frac{292 \times 100}{76.7} \times \frac{105}{100} = 400 \text{ samples}$$

In reality, 403 pork samples were taken and 332 *E. coli* isolates were isolated that met the statistical requirements in the sample size.

3.1.5 Study period

The field study allocated for sampling and sample analysis and for the questionnaire surveys was carried out from November 2006 to May 2007.

3.1.6 Sample analysis location

The laboratory work was conducted at the National Centre for Veterinary Hygiene Inspection (VHI), the Department of Animal Health (DAH), MARD of Vietnam, coded VILAB 059/ISO/IEC guide 17025: 2005, accredited by the Vietnamese Bureau of Accreditation (VBoA).

3.2 Methodology

3.2.1 Sampling and data collection

Sample collection

The population was divided into 14 groups corresponding to 14 administrative districts. 20 % of the total 232 communes/wards (called as commune) of all 14 districts were selected at random and subsequently 20 % of the total communes from each district. A specified number of samples were collected systematically corresponding to the proportion of communes of each district in the whole population. Number of pork shops per commune varied from five to six but there were only 3 pork shops selected systematically for sampling. The number of samples taken from each shop was typically 3; the actual sample numbers ranged from 2 to 4. Samples were taken systematically from every pork shop of every commune. The distribution of samples in districts (Table 4) and the method for sample collection (Chart 1) are as shown below.

Table 4: Number and distribution of samples in the districts of Hanoi area

District	No. of communes	Communes selected	No. of shops selected	Numbers of samples
Ba Dinh	14	3	9	24
Cau Giay	8	2	6	14
Dong Anh	24	5	15	42
Dong Da	21	4	12	37
Gia Lam	22	4	12	38
Hai Ba Trung	20	4	12	35
Hoan Kiem	18	4	12	31
Hoang Mai	14	3	9	24
Long Bien	14	3	9	24
Soc Son	26	5	15	45
Tay Ho	8	2	6	14
Thanh Tri	16	3	9	28
Thanh Xuan	11	2	6	19
Tu Liem	16	3	9	28
Grand Total	232	47	141	403

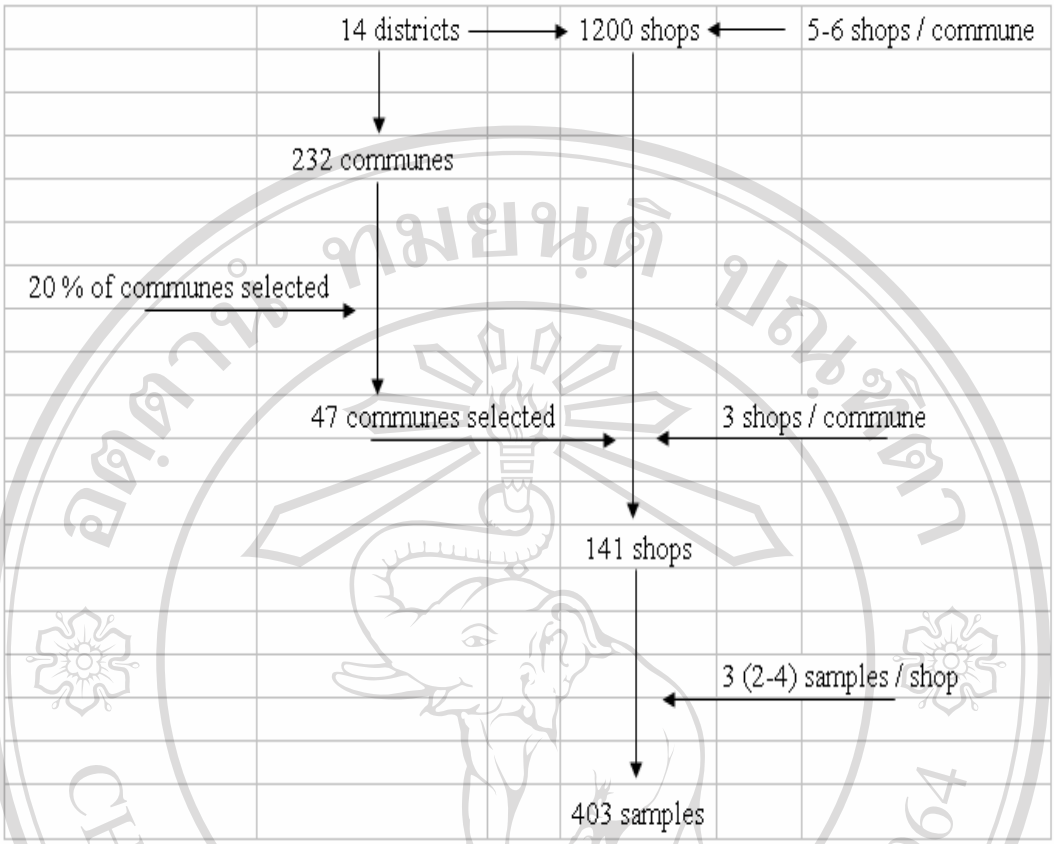

Chart 1: Chart showing the way for sample collection

The samples were collected in the morning, fifteen samples per time, twice per week for consecutive 14 weeks; not repeated. The materials used for collecting the samples were sterile plastic bags of a size of 18 cm x 25 cm. Information relating to the samples was recorded. After taking the samples they were stored in spongy boxes with ice at a temperature between 0 to 4°C. The samples were immediately transported to the laboratory and were kept intact until they were aseptically opened in the laboratory at the beginning of the examination (ISO 7218: 1996).

Data collection

There were two kinds of questionnaire surveys, one was to identify risk factors associated with antimicrobial resistance of *E. coli* isolated from pork relating to the

meat selling practice and the other one was to evaluate the pig husbandry practice by using designed questionnaires (appendix 2).

The questionnaire survey on selected factors relating to the pork selling practice included 141 questionnaires which were done simultaneously with the sampling of 141 pork retailers. The questionnaire survey on selected factors relating to the pig husbandry practice included 120 questionnaires. 80 questionnaires were done with 80 pig farmers in 20 communes in 5 suburban districts in Hanoi and 40 questionnaires were done with 8 communes of 4 districts of 4 neighboring provinces. The data collection was carried out simultaneously with the sampling.

The selection criteria for 'a pig farm' used in this study was that it had to be any farm or any household rearing pigs for selling pork to the Hanoi market in the five suburban districts of the Hanoi area or in the four neighboring provinces mentioned above. The size of a pig farm was based on the number of pigs for pork production in the current survey.

The list of communes in the five suburban districts recruited for the questionnaire survey which was related to the pig husbandry practice was the same list for collecting pork samples. Number and distribution of the questionnaires relating to the pig husbandry in these districts is shown in table 5.

Table 5: Number and distribution of questionnaires in five Hanoi suburban districts

No.	District	No. of communes	Communes selected	No. of questionnaires
1	Dong Anh	24	5	20
2	Gia Lam	22	4	16
3	Soc Son	26	5	20
4	Thanh Tri	16	3	12
5	Tu Liem	16	3	12
Grand Total		104	20	80

The selection of the four provinces for the questionnaire survey on the pig husbandry was based on the statistics that the pork sold to Hanoi market was highest from these provinces annually. At each province the study was carried out only for one selected district and two selected communes within the district that sold the highest amount of pork to the Hanoi market. Five questionnaires were collected from each selected commune.

The questionnaire for evaluating the pig husbandry practice (appendix 2a) included relevant information such as the rearing model, the farm size, the usage of antimicrobials in pig rearing and questions related to the assessment of the farmer's awareness on antimicrobial resistance and the farmers' profiles.

The questionnaire for evaluating the pork selling practice (appendix 2b) included relevant information such as the origin of meat, the amount of pork sold daily, the wrapping during the transport, the types of meat sold at the same shop and the meat retailers' profiles. The questionnaire surveys were performed as in chart 2.

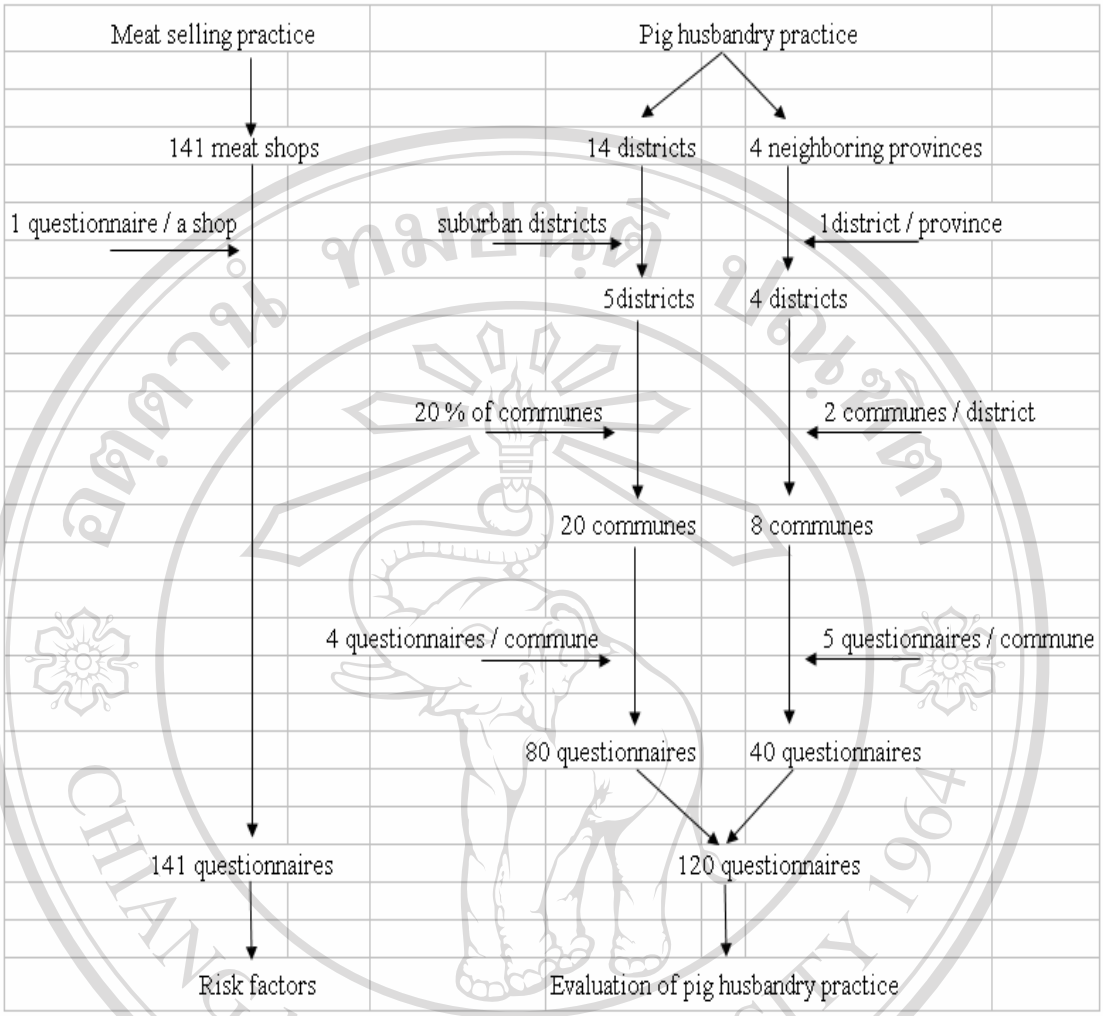

Chart 2: The data collection procedure for the analysis of the potential risk factors associated with antimicrobial resistance and evaluation of the pig husbandry practice

3.2.2 Methods for the sample analysis

Test for detecting the presumptive *E. coli* in fresh meat (ISO 7251: 2005)

- Step 1: Preparation of test portion (ISO 6887-2: 2003)

  The preparation of the test portion was performed in the vicinity of a flame with sterilized equipment and tools. Using a knife and scissors, an one cm

wide strip was cut along the length of the meat piece. Then this strip was cut into small pieces and was put into the stomacher bag.

- Step 2: Preparation of the initial suspension

  At first: 50 g of a sample was homogenized by a stomacher machine and diluted in 450 ml Peptone Water (PW).

  At the second: 10 ml of the initial suspension was mixed with an equal volume of a double strength LST tube.

- Step 3: Incubation of the selective enrichment medium (LST broth)

  The above double strength LST tubes were incubated at 37 ^{o}C for 24 h ± 2 h for examining gas. In case of no gas production the incubation was continued for up to 48 h ± 2 h.

- Step 4: Incubation of the selective medium (EC broth)

  A sampling loop was taken from the selective enrichment medium observed with gas and opacity and was inoculated into a tube of the EC broth, then incubated at 44 ^{o}C for 24 h ± 2h for gas production. If, at this step, no visible gas appeared in the EC broth, the incubation was continued for up to 48 h ± 2 h.

- Step 5: Testing for the indole production

  A sampling loop was taken from the EC broth observed with gas and inoculated into a tube of indole-free PW preheated at 44 ^{o}C, then incubated at 44 ^{o}C for 48 h ± 2h and the results were read by the indole test with Kovac's reagent (with red color in the alcoholic phase).

Interpretation: One sample was considered to be a presumptive *E. coli* positive when gas produced in the tube of the EC broth and the indole produced in the tube of indole-free PW at 44 ^{o}C for 48 h ± 2 h.

Confirmed test for *E. coli* (FAO, 1992)

- Step1: Isolation on the selective medium L-EMB
  Some portions of PW culture were streaked onto the surface of the selective medium L-EMB, it then incubated at 35 ^{o}C for 18-24 h, was examined for typical *E. coli* colonies (colonies having a dark center with or without metal sheen).

- Step 2: Inoculation on PCA slants
  The top of one well-isolated colony from the L-EMB was touched with a swab, then swirled it into one ml of 0.85 % sterile saline to create a cell suspension. The surface of the PCA slant was covered with the cell suspension, then incubated it at 35 ^{o}C for 18-24 h, then stored it at a temperature not higher than +4 ^{o}C for Gram stain, IMViC biochemical tests and antimicrobial susceptibility testing.

- Step 3: Gram stain (Gram-negative non spore-forming rods)

- Step 4: IMViC biochemical tests
  The top of colonies from the PCA slant was touched with a swab, then swirled into one ml of 0.85 % sterile saline to create a cell suspension. The surface of Simmons Citrate agar was covered with the cell suspension and each of the following media with a loop full of it: one tube 1 % tryptone broth, two tubes MR-VP broth, then incubated them for 48 h at 35 °C.

Interpretation: A presumptive *E. coli* that give the results of Gram-negative non spore-forming rods and IMViC patterns of "++--" or "-+--" was confirmed to be *E. coli*.

Method for antimicrobial susceptibility testing (Kirby-Bauer disk diffusion method)

- Step 1: Purification of isolates (Soomro, 2002)
  The top of colonies from the PCA slant was touched with a swab, and then swirled into one ml of 0.85 % sterile saline to create a cell suspension. The surface of the medium MacConkey agar was streaked with a loop, then inoculated for 18-24 h at 37 ^{o}C and examined for typical *E. coli* colonies (smooth, circular pink colonies with spreading growth).

- Step 2: Reconstitution and preservation of isolates (Alonso et al., 1999)

  Step 2.1: The top of one well-isolated colony in the medium MacConkey agar was touched with a swab, and then swirled into one ml of 0.85 % sterile saline to create a cell suspension. The same swab was used to cover the surface of the Nutrient Agar (NA) plate. Then it was incubated at 37 ^{o}C for 18-24 h and examined for typical *E. coli* colonies (small, colorless and yellowish white, circular, smooth colonies with entire edge).

  Step 2.2: Preservation for further study

  The top of one well-isolated colony in the NA was touched with a swab, then swirled into one ml of the BHI with 50 % glycerin and stored at -70 ^{o}C for further study (if possible).

- Step 3: Antimicrobial susceptibility testing by the standard Kirby-Bauer disk diffusion method following guidelines provided by the NCCLS (2000).

- Step 3.1: The top of each of five well isolated colonies in the NA was touched with a loop and the growth was transferred into a tube containing four to five ml of a Tryptic Soy Broth (TSB). The TSB was incubated at 35 ^{o}C for 2-6 h until it achieved the turbidity of the 0.5 McFarland standard (the turbidity of

this broth culture was adjusted with sterile saline to obtain a turbidity optically comparable to that of the 0.5 McFarland standard which was equal to a suspension containing approximately 1 to 2 x 10^8 cfu/ml for *E. coli* ATCC® 25922).

Step 3.2: The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. The lid may leave ajar for three to five minutes to get the suitable moisture before applying the drug impregnated disks. The drug impregnated disks used (Oxoid) which contained tetracycline (30 µg), oxytetracycline (30 µg), doxycycline (30 µg), trimethoprim (5 µg), sulphonamides (300 µg), streptomycin (10 µg), neomycin (30 µg), gentamicin (10 µg), ampicillin (10 µg), cephalothin (30 µg), norfloxacin (10 µg), orfloxacin (5 µg) were placed, individually, on the inoculated agar plate surface then they were incubated at 35 ^oC for 16-18 h. When applying antimicrobial impregnated paper disks on the surface of a Mueller-Hinton agar plate, antimicrobials in these disks diffused into the medium and inhibited the development of *E. coli* around paper disks with the halogens corresponding to the susceptibility of the *E. coli* to selective antimicrobials. Diameters of the halos were measured to mm. According to the diameter, *E. coli* isolates were classified into: resistant, intermediate and susceptible (Appendix 1).

The method for the analysis of sample is summarized in chart 3.

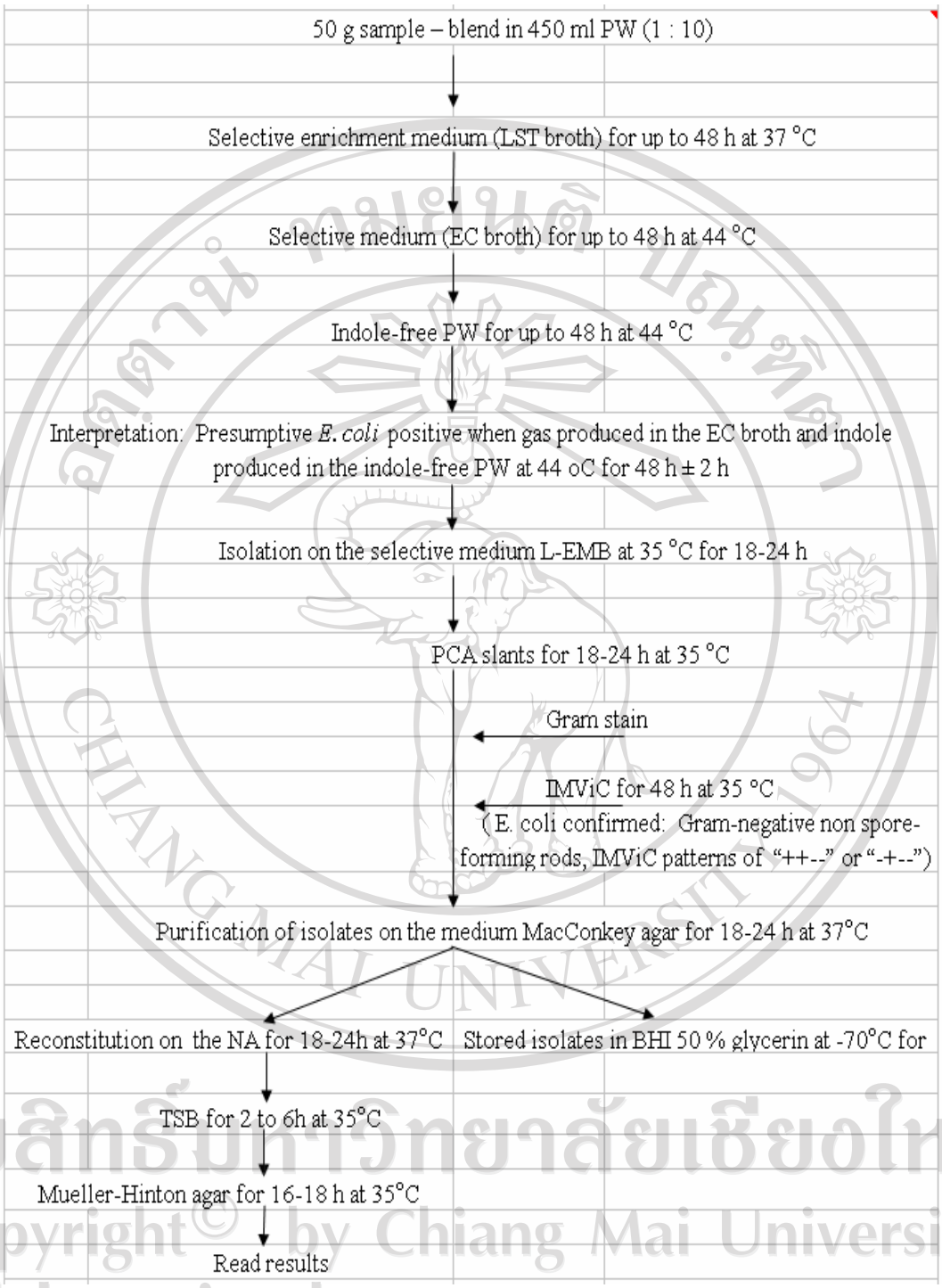

Chart 3: Summarizing the steps of the method for the sample analysis

3.2.3 Data processing

The Microsoft Excel was used to store the database.

The statistical software packages for Window which were used for the data analysis were SPSS version 11.5 and EpiCalc 2000 version 1.02.

Descriptive statistics: Use chart or/and percentage with 95 % CI to describe the data.

Inferential statistics: Chi-square test was used to assess the significantly statistical association between antimicrobial resistance of *E. coli* isolated from retail fresh pork with regard to potential risk factors (when the expected frequency in any cell is less than 5, Fisher's Exact test was used). The two sided level of significance was set at p-value ≤0.05.

Association between a potential risk factor and an antimicrobial resistance proportion was expressed by the Odds ratio (OR) with 95 % CI in there the OR is calculated from the 2–by–2 contingency table as shown in table 6.

Table 6: 2-by-2 table of results of each factor for the calculation of the Odds ratio

		Disease		**Total**
		(+)	**(-)**	
Exposure	(+)	a	b	a + b
	(-)	c	d	c+ d
Total		a+ c	b+ d	a+ b + c + d

OR = (a/b)/(c/d)=ad/bc