III. MATERIALS AND METHODS

3.1. Study design

3.1.1. Study site

Hanoi, the capital city, is located in the Red River Delta in North Vietnam, is comprised of fourteen districts, five suburb and nine urban districts.

Table 4. Study site and distribution of samples

No.	Districts	Numbers of samples by season		Total number of samples
		Winter	Spring	
1	Gia Lam	25	20	45
2	Soc Son	13	16	29
3	Dong Anh	16	17	33
4	Tu Liem	16	12	28
5	Thanh Tri	7	13	20
6	Cau Giay	8	10	18
7	Long Bien	11	16	27
8	Hoang Mai	9	11	20
9	Tay Ho	5	6	11
10	Thanh Xuan	12	10	22
11	Hai Ba Trung	4	5	9
12	Ba Dinh	1	4	5
13	Hoan Kiem	5	8	13
14	Dong Da	5	5	10
	Total	137	153	290

Climatically, Hanoi falls in the subtropical zone, influenced by tropical humid monsoon. There are four distinct seasons, namely spring (February-April), summer (May-July), autumn (August-October) and winter (November-January). It can also be divided into two: the rainy (May-September) and the dry (October-April) season. The average temperature in summer is 29.2^0C, in winter 17.2^0C and for the whole year 23.2^0C. There are about 114 rainy days a year with a mean rainfall of 1,800mm/year (Vietnamtourism, 2005).

Table 4 shows the study site and the distribution of samples. The study was conducted in the five suburb districts namely Gia Lam, Soc Son, Dong Anh, Tu Liem, and Thanh Tri and in the nine urban districts Cau Giay, Long Bien, Hoang Mai, Tay Ho,Thanh Xuan, Hai Ba Trung, Ba Dinh, Hoan Kiem, and Dong Da of Hanoi.

3.1.2. Study population and sample size determination

Permanent regular markets in all above districts were selected as sampling sites; meat shops in the markets were defined as study units. However, only a certain percentage of these shops, which were sufficed to a statistically minimum required number of samples, were selected. The number of samples was estimated by using the computer program Win-Episcope 2.0. An expected prevalence of antibiotic residues at detectable levels in pork was 10% (An *et al.*, 2002; Thuận *et al.*, 2003). Based on the expected prevalence of 10%, the study population (N) in 14 districts of 1200, 95% confidence level, and 5% of accepted error, the required sample size (n) was 125 (10.33% of the population). To satisfy the requirement on sample size (one-tailed) for estimating difference between two percentages (expected proportion in group 1 as 5%, group 2 as 15%; 95% confidence level, and power 85%), the sample size was estimated as 127 samples for one time and 254 for two times of sampling. In actual, 290 samples altogether were collected by two times of sampling that satisfied both requirements.

3.2. Methods

3.2.1. Sampling and data collection

The sampling was conducted in two seasons, namely early winter and early spring. Meat shops were randomly selected from each market. The sampling was not repeated for previously selected samples. In other words, a shop selected by the first sampling round was then not selected in the second round. This means that 290 different shops were selected. From each shop, one muscle sample of approximately 300 to 400 grams was collected, wrapped in P.E. bag and put in a cool box with ice. Samples were then transported to the laboratories and stored in freezers at a temperature of no higher than -18^0C until analysis. Number and distribution of samples are shown in table 4. Relevant information such as location of the market, origin of meat, type of abattoir, product(s) offered on the same shop, and shop-owner's profile was also obtained simultaneously at the time of sampling by using a questionnaire (Appendix 1). This collected information would be subsequently used in the analysis of risk factor.

3.2.2. Methods for analysis

3.2.2.1. Microbiological inhibition test

This method was used as a screening test. All samples were analyzed by using the microbiological inhibition test with *Bacillus cereus* ATCC 11778 as a reference bacterium, oxytetracycline discs (Mast Diagnostics 0.5 µg/disc) as control, on agar test pH 6 (Merck) (SOP RES 31 V.8, 2002; Myllyniemi *et al*., 2001; Nouws *et al*., 1998). The sterile bottles of medium were melted and sterilized in an autoclave at 121^0C for 15 minutes; subsequently placed in a waterbath at 55^0C and left for at least 30 minutes until they reached the temperature of the waterbath. Appropriate volumes of the *Bacillus cereus* spore suspension were added to the medium, gently mixed and poured into 90mm-diameter sterile Petri dishes on a leveling platform with 5ml/plate.

Muscle samples were removed from the freezer and placed at room temperature for up to 20 minutes. An 8mm-diameter cylindrical core from each sample was cut using a stainless cork borer. The core was subsequently cut into slices of 2mm thickness using a sterile scalpel blade. Two slices from each sample were placed opposite each other on a plate using forceps; a positive control disc was placed in the center of the plate. The plates were incubated at 30^0C for approximately 18 hours. Plates were read against a black background with a light from underneath. The zones of inhibition given by the tissue slices and control discs were measured to the nearest mm using a ruler. Positive results were indicated by the complete inhibition of growth around both meat slices in a zone of 12 mm diameter or greater (the annular zone not less than 2 mm wide). Negative results were indicated by no inhibition of growth around the meat slices. The inconclusive samples, which showed the annular inhibition zone less than 2 mm wide or incomplete, were then, together with the positive ones, analyzed by the high performance liquid chromatography (HPLC) mentioned below.

3.2.2.2. Analysis by HPLC

Samples that were previously considered as inconclusive results, as well as samples which were indicated as positive by microbiological inhibition test were subsequently analyzed by HPLC. These samples underwent three principal stages in the sample preparation (i) homogenization and extraction of the sample residues by EDTA/Mc Ilvain buffer; (ii) precipitation of proteins using trichloroacetic acid and filtration; and, (iii) cleanup on solid-phase extraction cartridges C_{18}. Tetracyclines are separated on a C_{18} stationary phase and detected by UV absorption at 355 nm. The amount of tetracycline is calculated by interpolation from a calibration curve determined for each of the three compounds: oxytetracycline, tetracycline and chlortetracycline, taking into account the calculated recovery. The detailed procedures are mentioned in the standard of Agence Française de Sécurité Sanitaire des Aliments (AFSSA) for “Determination of Tetracycline residues in kidney and muscle by high performance liquid chromatography” (Appendix 2).

3.2.3. Further data processing

A sample that was indicated as a positive result by either above-mentioned methods was defined as a positive result in this study. Data were described using percentage and range. The inferential statistics were Fisher's exact test for identification of risk factors. A statistical software package used for analyzing data was NCSS version 2000. A confidence level of 95% (α=0.05) was defined. Odds ratio was calculated as given in table 5.

Table 5. 2-by-2 table of results of each factor to calculate odds ratio

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

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

Quantitative data obtained from HPLC analysis, and the data collected from the questionnaire survey were analyzed using descriptive statistics such as percentage, mean and range.