CHAPTER 3
MATERIALS AND METHODS

3.1. Study design

3.1.1 Study design

This is a cross-sectional study on microbiological contamination of fresh minced pork in retail markets with a questionnaire survey obtaining from butcher shops in Hanoi from 9 urban districts.

3.1.2 Study site

The study site covers the city of Hanoi involving Hanoi's wet markets. There are nine urban districts namely Caugiay, Longbien, Hoangmai, Tayho, Thanhxuan, Haibatrung, Badinh, Hoankiem, and Dongda in Hanoi. The study was conducted in all districts for collecting fresh minced pork from retail meat shops and also obtaining data from the questionnaire survey.

3.1.3 Study population and sample

The study population are permanent markets from the nine urban districts of Hanoi city. But only a certain percentage of these shops which were sufficient to a statistically required minimum numbers of samples were selected. According to the Hanoi Sub-Department of Animal Health experts (CCTYHN, 2008) there are about 600 minced meat shops in 9 urban districts.

3.1.4 Sample size

The sample size for determining the prevalence of *Salmonella* spp.: The hypothesis is that the expected prevalence of *Salmonella* spp. is 50%. using the Win-Episcope 2.0 with population size (N) of 600 minced meat shop with a 95% confidence level and 5% of accepted error. The required sample size for determining the prevalence of *Salmonella* spp. which was isolated from fresh minced pork in Hanoi, Vietnam is 251.

The sample size for determining the prevalence of *Salmonella* spp. covered the required sample size for counting the APC and *E.coli* from fresh minced pork. A total of 251 fresh minced pork samples, 98 of these samples were also systematically chosen to test for the APC, *E. coli* count.

3.1.5 Study period

The field study time combined with sampling and sample analysis and the questionnaires survey ranged from the middle of November, 2008 to April, 2009.

3.1.6 Research place location

The laboratory of the National Centre for Veterinary Hygiene Inspection No1, Department of Animal Health of Vietnam coded VILAB 059/ISO/IEC Guide 17025.

3.2 Methodology

3.2.1 Sampling technique

3.2.1.1 Sampling collection

The population was divided into 9 groups corresponding to 9 administrative districts. As sampling collection was used the stratified sampling technique from 20% of 128 wards of all 9 districts (29 wards) based on the alphabet order of ward list of every district. The number of shops selected from each ward was 8- 9. One sample was taken from each shop. The distribution of the samples in the districts (Table 9) and the method for the sample collection (Figure 2) were shown as below.

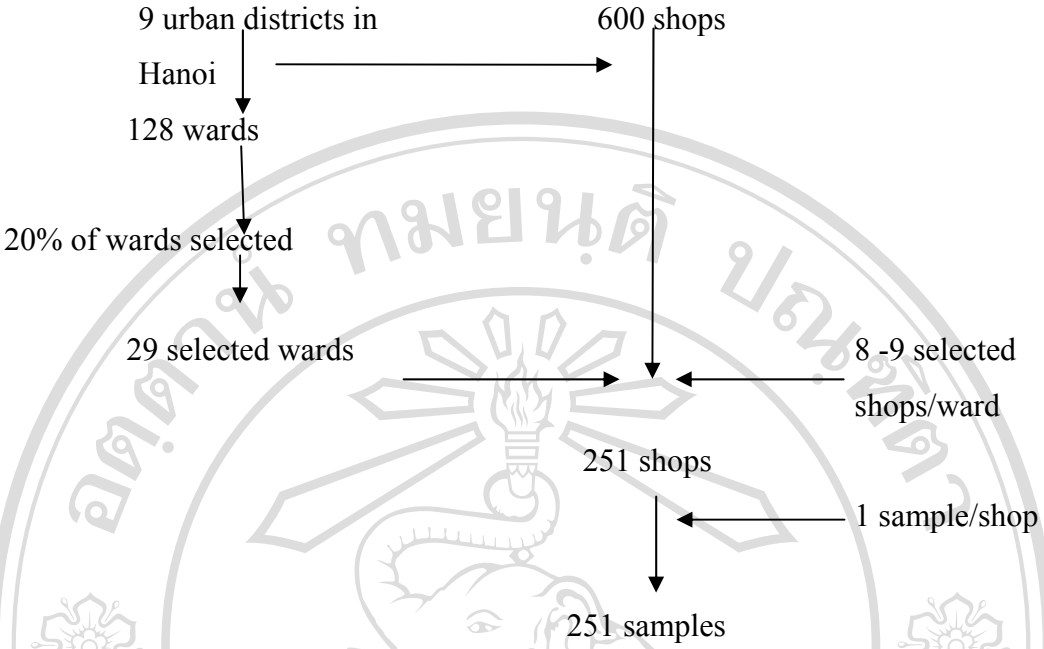


Figure 2: Chart showing the channels for collecting samples.

Table 9: Number and distribution of samples in 9 urban districts of Hanoi

Order	District	Numbers of wards	20% of wards selected	Numbers of samples selected (*Salmonella* spp.)	Numbers of samples selected (APC, *E.coli* count)
1	Badinh	14	3	27	9
2	Caugiay	8	2	18	5
3	Dongda	21	4	36	13
4	Haibatrung	20	4	32	10
5	Hoankiem	18	4	32	12
6	Hoangmai	14	4	36	15
7	Longbien	14	3	28	8
8	Tayho	8	2	18	6
9	Thanhxuan	11	3	24	20
Grand Total		128	29	251	98

3.2.1.2 Types of samples:

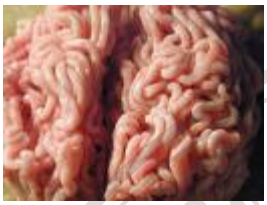


Figure 3: Type of sample: a) minced pork; b) meat grinder of butchers shop

Amount of sample: Approximately one hundred fifty gram of fresh minced pork was purchased through random sampling from butcher shops at 11.00 am - 12.30 am at noon. Fresh minced pork was packed into an 18 cm x 25 cm sterile polypropylene plastic bag. The detail information of the fresh minced pork sample was marked on the surface of the plastic bag.

For the transportation and the storage of the sample the guideline of ISO 7218:1996 (E) (Microbiology of Food and Animal Feeding Stuffs – General Rules for Microbiological Examinations) was applied. Samples were stored in spongy boxes with ice at a temperature between 0^{o}C - 4^{o}C and were transported to the laboratory. The samples awaiting examination were kept refrigerated at temperatures of 0^{o}C - 4^{o}C for a maximum of 24h. The samples needed to be kept intact until they were aseptically opened in the laboratory at the beginning of the examination.

3.2.2 Data collection

For the identification of some risk factors associated with the contaminated *Salmonella* spp., *E. coli* and the APC isolated from butcher shops a designed questionnaire was used and displayed in Appendix 1.

A questionnaire survey was conducted, simultaneously, with the sampling of 251 pork retailers to assess associations between the routine practices of fresh minced pork handling and the microbiological contamination in retail fresh minced meat.

3.3 Methods for the sample analysis

3.3.1 Isolation of *Salmonella* spp. (Based on **ISO 6579:2002)**

3.3.1.1 Sample preparation and pre- enrichment

Twenty five gram of fresh minced meat was transferred into a stomacher bag, then, 225 ml Buffered Peptone Water (BPW) were added and the sample was homogenized by a stomacher lab-blender. The homogenized sample was incubated at 37^{O}C for 18 ± 2h.

3.3.1.2 Selective enrichment

The pre-enrichment broth was mixed and 0.1 ml of pre-enrichment was transferred to 10 ml pre-warmed Rappaport Vasiliadis medium (RVS) enrichment broth, incubation at 42^{O}C for 24h ± 3h.

3.3.1.3 Plating and identification

After the incubation for 24 h a loopful of the RVS enrichment culture was transferred and streaked onto the surface of Xylose Lysine Tergitol 4 agar (XLT4) and Brilliant- green Phenol-red Lactose Sucrose Agar (BPLS), separately. The plates were incubated in an inverted position at 37^{O}C for 24 h. After the incubation the plates were checked for growth of typical *Salmonella* colonies. Typical colonies were defined as follows:

On the BPLS media, *Salmonella* colonies were reddish color and translucent colony.

On the XLT4 media, *Salmonella* colonies were black centered with lightly transparent zone of reddish color due to color change of the indicator (*Salmonella* H_2S negative variants, e.g. *Salmonella* paratyphi A were pink with a darker centre. Lactose – positive *Salmonella* were yellow with or without blackening.

3.3.1.4 Confirmation

Nutrient agar: Selection of colonies for confirmation

For confirmation up to five typical or suspect colonies per petri dish on the selective medium (XLT4 and BPLS) were transferred and tested by biochemical tests. The selected colonies were streaked on the surface of pre-dried nutrient agar plates and incubated at 37^{O}C for 24 h ± 3 h using a method that allowed the development of isolated colonies.

Biochemical Confirmation

After the incubation the pure colonies on a nutrient agar were picked up and inoculated into a Triple Sugar Iron (TSI) slant, Motility Indole Lysine Agar (MIL) and Urea slant**.** All inoculated biochemical media were incubated at 37^{O}C for 18 – 24 hours.

Triple Sugar Iron Agar (TSI Agar)

The agar slant surface was streaked and incubated at 37^{O}C ± 1^{O}C for 24 ± 3 hours. The results were recorded as listed in Table 10.

Table 10: Observation of TSI Agar for the presence of *Salmonella* spp.

Area of reaction	**Result**	**Interpretation**
Butt	Yellow	Glucose positive (glucose utilized)
	Red or unchanged	Glucose negative (glucose not utilized)
	Black	Formation of hydrogen sulfide
	Bubbles or cracks	Gas formation from glucose
Slant surface	Yellow	Lactose and/or sucrose positive (lactose and/or sucrose utilized)
	Red or unchanged	Lactose and/or sucrose negative (neither lactose nor sucrose utilized)

Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and a formation of hydrogen sulfide (blackening of the agar). When lactose-positive *Salmonella* strains were isolated the TSI agar slant was yellow. Thus, preliminary confirmation of *Salmonella* cultures was not based on the results of the TSI agar test only.

Urea agar

The agar slant surface was streaked and incubated at 37 ± 1˚C for 24 ± 3 hours and the examination was done at intervals.

If the reaction was positive the splitting of urea liberates ammonia which changed the color of phenol red to rose pink and later to deep cerise (moderate red) was noticed. The reaction was often apparent after 2 to 4 hours.

Motility Indole Lysine Agar (MIL)

A tube containing 5 ml of MIL broth was inoculated with the suspect colony and incubated at 37 ± 1˚C for 24 hours. After reading the lysine decarboxylase, motility and lysine deaminase reactions 1 ml of Kovacs reagent was added and gently shaken to determine the indole reaction. The formation of a red ring indicated a positive Kovacs reaction. A yellow-brown ring indicated a negative reaction.

The Medium for the Voges-Proskauer (VP) reaction

A loopful of the suspect colony was suspended in 3 ml of the VP medium in a sterile tube and incubated at 37 ± 1˚C for 24 ± 3 hours. After the incubation 2 drops of a creatine solution, 3 drops of an ethanolic solution of 1-napthol and then 2 drops of potassium hydroxide solution were added. The mixture had to be shaken after the adding of each reagent. The formation of a pink to a bright red color within 15 minutes indicated a positive reaction. In the case of *Salmonella* spp. the reaction had results as follows in Table 11:

Table 11: Interpretation of the biochemical test results of *Salmonella* spp.

Biochemical tests	*Salmonella* strains				
	S. Typhi	*S*. Paratyphi A	*S*. Paratyphi B	*S*. Paratyphi C	Other strains
TSI acid from glucose	+	+	+	+	+
TSI gas from glucose	-	+	+	+	+
TSI acid from Lactose	-	-	-	-	-
TSI acid from sucrose	-	-	-	-	-
TSI H_2S production	+	-	+	+	+
Urea hydrolysis	-	-	-	-	-
Lysine decarboxylation	+	-	+	+	+
Voges-Proskauer reaction	-	-	-	-	-
Production of indole	-	-	-	-	-

The steps of the *Salmonella* spp. analysis were concluded in the flow chart and are shown in figure 4

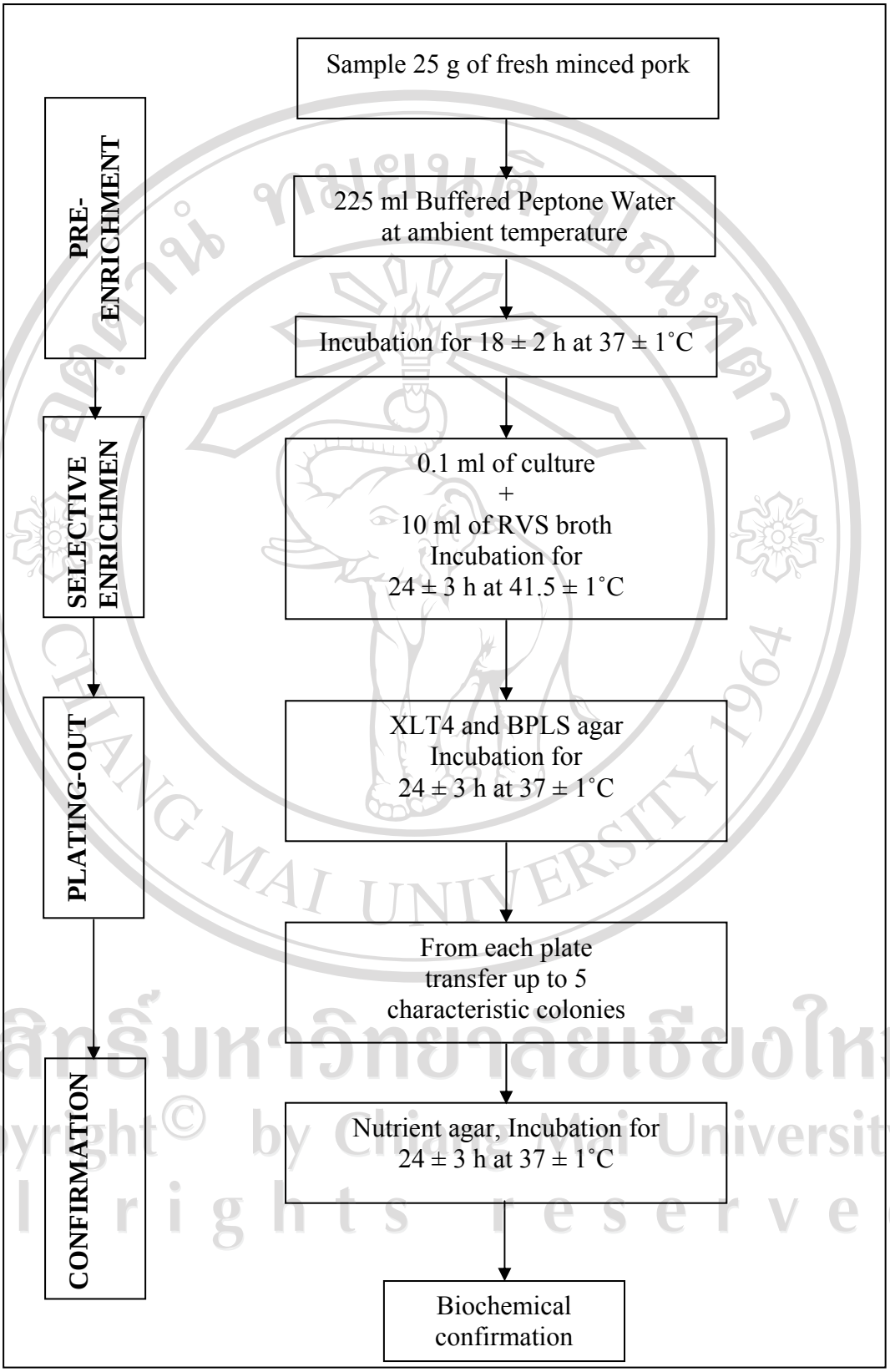


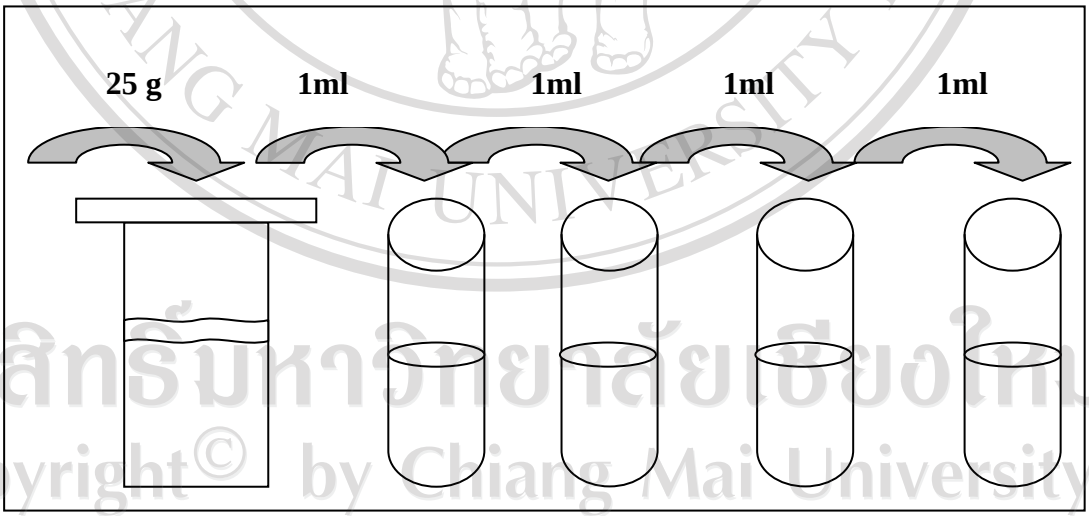
Figure 4: Flow chart of the isolation of *Salmonella* spp. by the conventional culture method (ISO 6579, 2002).

3.3.2 Total Aerobic Plate Counts - Based on the ISO 4833:2003 Standard Protocol

3.3.2.1. Procedures

1.1 Perform decimal dilutions (Preparation of the initial suspension according to ISO 6887-1) and inoculation.

For the microbiological analysis the analytical unit (25g) was added to 225 ml of 0.1% peptone water and homogenized in a stomacher (IUL instrument) for 1 minute at medium speed and room temperature. Serial decimal dilutions were done by transferring 1 ml of initial suspension (10^{-1}) into a tube containing 9 ml of 0.1% peptone water. Then the mixture was homogenized to make a 10^{-2} dilution. To prepare the further decimal dilutions, 1 ml of the 10^{-2} dilution was transferred into a tube containing 9 ml of 0.1% peptone water to make a 10^{-3} dilution. These operations were repeated by using a new sterile pipette to obtain 10^{-4} to 10^{-6} dilutions, as shown in Figure 5.



Volume (ml)	225 ml	9 ml	9ml	9 ml	9 ml	9 ml
Dilution	1/10	1/10	1/10	1/10	1/10	1/10
Total Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}

Figure 5: Preparation of the initial suspension according to ISO 6887-1

1.2 Mix the dilutions using a vortex mixer for 5 to 10 seconds.

1.3 If appropriate and possible select only two critical dilution steps for the inoculation of the Petri dishes that will give colony counts of between 15 and 300 colonies per plate. 1 ml of appropriate diluents will be transferred to 2 sterile Petri dishes.

1.4 Pour about 12 ml to 15 ml of the plate count agar (PCA) at 44 ^{o}C to 47 ^{o}C into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into the dishes will not exceed 45 min.

1.5 Carefully mix the inoculum with the medium by rotating the Petri dishes and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface.

1.6 Invert the Petri dishes and place in stacks of six or less. Transfer them to an incubator at 30^{o}C ± 1°C for 72 hours ± 3 hours.

2. Counting of colonies

The precision data is evaluated for dishes containing more than 15 colonies and fewer than 300 colonies. The data below 15 colonies and more than 300 colonies may be used as an estimated colony counts.

3. Calculation

$$\text{Number of micro-organisms} = \frac{\sum c}{(n1+0.1n2)d}$$

$\sum c$ = the sum of colonies counted

n1 = the number of dishes retained in the 1st dilution

n2 = the number of dishes retained in the 2nd dilution

d = the dilution factor corresponding to the first dilution

Round the result calculated to two significant figures. Report the result as the Total Aerobic Count per g test material.

3.2.3 Method for the Enumeration of ß-glucuronidase-positive *Escherichia coli* (ISO 16649 -2:2001)

3.2.3.1 Preparation of the initial suspension according to ISO 6887-1

1.1 Transfer 1 ml of the initial dilution (10^{-1}) to a sterile Petri dish of approximately 90 mm diameter. Inoculate two plates per dilution. Repeat the procedure with the further decimal dilutions.

1.2 Mix the dilutions using a vortex mixer for 5 to 10 seconds.

1.3. Pour approximately 15 ml of the (Tryptone-bile-glucoronic) TBX medium, previously cooled at 44^{O}C to 47^{O}C, in the water bath into each Petri dish. Mix the inoculum with the medium and allow the mixture to solidify with the Petri dishes standing on a cool horizontal surface. Invert the inoculated dishes so that the bottom is uppermost and place them in an incubator set at 44^{O}C for 18 h to 24 h.

3.2.3.2 Counting the Colony Forming Units (CFU)

Count the typical CFU of ß-glucuronidase-positive *E. coli* in each dish containing less than 150 typical CFU and less than 300 total (typical and non-typical) CFU. The typical CFU of ß - glucuronidase-positive *E. coli* is a blue colony.

If the dishes contain 0 typical CFU they should be taken into consideration for counting the low- number calculation methods recommended below.

3.2.3.3 Calculation

Normal cases

$$N = \frac{\sum a}{V(n1+0.1n2)d}$$

∑a is the sum of the CFU counted on all the dishes retained from two successive dilutions, at least one of which contains a minimum 15 blue CFU;

n1 is the number of dishes retained at the first dilution

n2 is the number of dishes retained at the second dilution

V is the volume of inoculum, in milliliters applied to each dish.

d is the dilution factor corresponding to the first dilution retained where the directly inoculated test sample is retained.

Calculation of low numbers

a) If the two dishes at the level of the initial suspension or of the first inoculated or retained dilution contain less than 15 blue CFU, calculate N_E, the number of CFU of ß - glucuronidase-positive *E. coli* present in the test sample, like the arithmetical mean from two parallel plates using the following equation:

$$N_E = \frac{\sum c}{V*n*d}$$

$\sum c$ is the sum of the blue CFU counted on the two dishes

V is the volume of the inoculum applied to each dish;

n is the number of dishes retained (n=2 in this case);

d is the dilution factor to the initial suspension or the first inoculated or retained dilution (d=1 in the case (liquid products) where the directly inoculated test sample is retained). Express the result as follows:

Estimated number of ß-glucuronidase-positive *E. coli* per gram: N_E =Y.

b) If the two dishes at the level of the initial suspension of the first inoculated or retained dilution do not contain any blue CFU, express the result as follows

Less than 1/d of ß-glucuronidase-positive *E. coli* per gram

d is the dilution factor of the initial suspension or the first inoculated or retained dilution.

c) If for the two dishes for the first dilution d_1 the total number of blues and non typical CFU is higher than 300 with visible blue CFU and if for the two dishes from the subsequent dilution d_2 contain less than 300 colonies no blue CFU can be counted, express the result as follows: less than $1/d_2$ and more than $1/d_1$ ß-glucuronidase-positive *E. coli* per gram.

d) If for the two dishes form the first dilution d_1 the total number of typical CFU and non typical CFU is higher than 300 without visible blue CFU, if for the two

dishes from the subsequent dilution d_2 containing less than 300 colonies, no blue CFU can be counted express the result as follows:

Less than $1/d_2$ CFU of ß-glucuronidase-positive *E. coli* per gram

d_2 is the dilution factor corresponding to dilution d_2

Method of calculation: Special cases

a) If the number of blue CFU is higher than 150 for the two dishes from the first dilution d_1, with a number of blue CFU 15 for the two dishes from the subsequent dilution d_2 :

If the number of blue CFU on each of the two dishes from dilution d_1 is within the range of 167 to 150 (the upper part of the confidence interval of a weighted mean is equal to 150), use the calculation method for the general case.

If the number of blue CFU on each of the two dishes from dilution d_1 is higher than 167 (upper limit of the confidence interval of a weighted mean equal to 150 CFU) only take into account the result of the counts of dilution d_2 and carry out a low number count.

b) Counting the blue CFU on each of the dishes from all the inoculated dilutions gives a number higher than 150,

More than 150/d ß-glucuronidase-positive *Escherichia coli* per gram

D is the dilution factor of the last inoculated dilution

c) In case that only the two dishes from the lowest dilution (highest concentration) contain less than 150 typical CFU, calculate the number N_E of ß-glucuronidase-positive *E. coli* present in the test sample as the arithmetical mean of the colonies counted on the two dishes, using the following equation.

$$N_E = \frac{\sum c}{V*n*d}$$

∑c is the sum of the blue CFU counted on the two dishes, of which at least one contains at the minimum 15 typical CFU.

N is the number of dishes retained (n=2 in this case).

v is the volume of inoculum

d is the dilution factor corresponding to the dilution retained.

3.2.4. Data processing

Microsoft Excel was used to store data. The statistical software package which was used for analyzing the data is SPSS version 11.5, STATA/Se 9.2. All statistical analysis was interpreted at a 5% level of significance.

Chart, percentage, mean and median were used to describe the prevalence, the microbiological contamination in terms of the APC, the *E. coli* count and the absence or the presence of *Salmonella* spp.

An univariate analysis was carried out and compared the proportion using the Pearson's Chi-square or Fisher's exact test to find a statistically significant difference of factors related to qualitative variables. *E. coli* counts were analyzed by non-parametric tests such as the Mann-Whitney test and the Kruskal-Wallis test. A T-test was performed to compare the mean of APC between 2 categories of risk factors and ANOVA was used to compare the mean among 3 or more categories of risk factors.

The association between a potential risk factor and the proportion of the *Salmonella* contamination was expressed by the Odds ratio (OR) with 95% CI. The OR is calculated from the 2 x 2 table as shown in table 12.

Table 12: Tables of results of each factor for the calculation of the Odds ratio

		Salmonellae		**Total**
		Positive	Negative	
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