CHAPTER III

MATERIALS AND METHODS

3.1 Study scope and design

This study targeted onto medium slaughterhouse scale (from 10 to 100 pigs per days) and local market, which might deliver high pork proportions and which are popular in Vietnam. The study detects *Salmonella* spp. prevalence and concentration on pig carcass at slaughterhouse and pork at market. *Salmonella* spp. prevalence was determined by using biological analyses based on ISO 6579:2002. *Salmonella* concentration in samples was enumerated by using 3-tube Most Probable Number (MPN) method. Study also identifies risk factors of *Salmonella* spp. contamination in the pork production chain (pig slaughterhouse and market). Potential risk factors were evaluated and tested in relation to *Salmonella* positive in pig carcasses and pork samples. The risk factors associated with *Salmonella* positive were considered statistically significance at p-value less than 0.05. Apart from this quantitative (checklist, questionnaire) and qualitative (in-depth interviews (IDI) and focus group discussions (FGD)) approaches were used to study perception and practice of different groups and actors along the pork chain on food safety and zoonoses.

Study design: Study was designed as a cross-sectional study.

3.2 Study location and duration

The study was carried out in Hung Yen province, Vietnam. Three pig slaughterhouses in three districts, namely Van Giang, Van Lam and My Hao, were selected (Figure 2). In one district, we selected 1 slaughterhouse and followed the products to 2-3 markets for sampling. In each studied site, we visited 4 times to take samples and collect data and information.

The laboratory works were performed at Department of Veterinary Hygiene, National Institute of Veterinary Research, Hanoi, Vietnam. Serotyping of isolated *Salmonella* was done at Institute of Meat Hygiene and Technology, Faculty of Veterinary Medicine, Freie Universität of Berlin, Germany.

Study duration: Study was carried out from December 2012 to May 2013.

Figure 1 Hung Yen province and three selected slaughterhouses (red points)

3.3 Material and methods

3.3.1 Sample collection

3.3.1.1 Sampling size

The average of slaughtered pigs per day was counted as a total population (N ~ 920) for sample size calculation (Appendix 2.1). We assumed a prevalence (p) of

Salmonella on carcass at slaughterhouse as 50%. It was estimated from previous studies of Takeshi et al. in 2009 (15.5%), Thai in 2009 (48.9%) and Le Bas et al. in 2006 (95.7%), with a 10% error rate (L) and 95% confidence interval (Z=1,96).

By using Win Episcope (2.0, 1998) program to calculate, sample size was equal 87 samples. We planned to collect sample at slaughterhouse and market, so the total number of samples was 174.

3.3.1.2 Sampling frame

Regarding accessibility in sampling, three selected slaughterhouse were sampled at 4 visits during the study period. Each visiting day, we took samples from both slaughterhouse and markets. At slaughterhouse, after splitting the carcass, swabs or belly tissue were randomly selected during slaughtering time. Carcass (swab or belly tissue) samples were collected up to 4 to 7 samples per visit. Slaughter workers sampling was done by swabbing two hands of one worker who had the last touch on the carcass just after splitting. Cutting boards (or surface of the splitting place) were swabbed after moving split carcass. The same visiting day, we followed 2 carcasses which were sampled at slaughterhouse to sample them again at market. Other pork samples were also taken from carcasses which were from the same slaughterhouse. Seller hands and cutting board swabs at pork shops were done the same way (2 hands of pork sellers and cutting board surface).

In 12 visits, we collected 63 carcass samples, 63 pork samples and other 48 environmental samples (cutting board at slaughterhouse, worker hands, seller hands and cutting board at market). Sample frame and collected samples are shown in Table 4. Pig slaughtering process was described in Appendix 2.2.

Table 1 Collected sample frame at pig slaughterhouses and pork markets

District	Slaughterhouse			Market			Total
	Carcass swabs	Cutting boards	Worker hands	Pork	Cutting boards	Seller hands	
Van Lam	**21**	**4**	**4**	**21**	**4**	**4**	**58**
D1-01	5	1	1	7	1	1	16
D1-02	6	1	1	5	1	1	15
D1-03	6	1	1	6	1	1	16
D1-04	4	1	1	3	1	1	11
Van Giang	**21**	**4**	**4**	**21**	**4**	**4**	**58**
D2-01	6	1	1	6	1	1	16
D2-02	5	1	1	5	1	1	14
D2-03	5	1	1	5	1	1	14
D2-04	5	1	1	5	1	1	14
My Hao	**21**	**4**	**4**	**21**	**4**	**4**	**58**
D3-01	6	1	1	6	1	1	16
D3-02	6	1	1	6	1	1	14
D3-03	6	1	1	5	1	1	14
D3-04	3	1	1	4	1	1	14
Total	**63**	**12**	**12**	**63**	**12**	**12**	**174**

3.3.1.3 Swab and pork sampling

i. Carcass sampling

After splitting, we used using sterile moisture gauze with a 100 cm^2 steel frame (10x10 cm) to swab at 4 sites on the medial of carcass. So, one swab carcass sample covered 400 cm^2 of surface. Belly tissue (including skin) was collected by cutting along the evisceration line at abdominal site after splitting carcass. The amount of skin was about 100 grams.

ii. Cutting boards and hand swab sampling

Swab samples on cutting boards or hands which were carried out at slaughterhouses and markets were following ISO 17604:2003 (ISO-17604, 2003). A

25 cm^2 frame (5x5 cm) was used to swab once on cutting boards' surface. Two hands of slaughter worker were swabbed on whole palm, fingers and between fingers.

iii. Pork sampling

Pork did not include organs such as heart, liver, and intestine. Sampling process was done following ISO 17604:2003 (ISO-17604, 2003). The amount of collected pork was approximately 200 grams to ensure representatively including skin, fat and lean tissues. Samples were marked with the date, time, shop and market for identification of sample code in laboratory. Pork samples were kept separately in an icebox at around 2 to 4°C (ISO-6579, 2002). Samples were then storage and transported to the laboratory within 6-12 hours after collection for analyses.

3.3.2 *Salmonella* detection and enumeration

3.3.2.1 Salmonella detection

We followed the protocol of ISO 6579:2002 (Microbiology of food and animal feeding stuffs - horizontal method for the detection of *Salmonella*) to detect *Salmonella* with slight modifications. The laboratory procedure for isolation and identification of *Salmonella* is described in Appendix 2.3.

i. Sample preparations and pre-enrichment

Pork or belly skin samples were weighed and placed into a sterile stomacher bag, Buffered Peptone Water (BPW; Merck KGaA, Germany) was added in a dilution of 1:10 (25 g pork was cut by sterile scissors and forceps into small pieces then put into a marked stomacher bag, then filled with 225 ml Buffered Peptone Water). The stomacher bag was then homogenized by a stomacher machine.

For swab samples (cutting boards, hands, carcasses), each swabbed sample was placed in a stomacher bag, BPW was added to a total volume of 100 ml (ISO-

17604, 2003). The stomacher bag was then homogenized by a stomacher machine.

All homogenate solutions were incubated at 37 ± 1°C for 18h ± 2h. In pre-enrichment step, *Salmonella* may be present in small numbers and often accompanied by considerably larger numbers of other Enterobacteriaceae or other bacteria. Pre-enrichment is necessary to assist isolation and therefore enhances the detection of low numbers of *Salmonella* or injured *Salmonella*.

ii. Selective enrichment

The incubated pre-enrichment broth was mixed and transferred to Modified Semi-solid Rappaport Vassiliadis (MSRV) and Muller Kauffmann Tetrathionate novobiocin broth (MKTTn). Three drops (~50 µl/drop) of pre-enrichment broth were given onto a surface of MSRV agar plate which was then incubated at 41,5 ± 1°C for 24 hours to 48 hours for selective enrichment step. Other 1 ml of incubated pre-enrichment broth was also mixed in 10 ml MKTTn then was incubated at 37 ± 1°C for 24 hours ± 3 hours.

iii. Plating out and identification procedures

After incubation for 24 hours (or up to 48 hours for MSRV plates), a loop of material from MSRV (at the edge of migration zone) and MKTTn broth were transferred by streaking separately onto the surface of XLT4 agar (Xylose Lysine Tergitol 4 agar) and BGA agar (Brilliant Green Agar). These agar plates then were incubated at 37 ± 1°C for 24 hours ± 3 hours.

Identification after incubation was done by examining the typical specific characteristics of *Salmonella* spp. colonies. On BGA agar plates, typical *Salmonella* colonies show pink color and surrounded by a red zone in the medium. On XLT4 agar plates, typical *Salmonella* colonies (H2S-positive) appear black or black-centered with a yellow periphery after 18 -24 hours of incubation. Upon continued incubation, the colonies become entirely black or pink to red with black centers. Colonies of H2S-

negative *Salmonella* strains appear pink-yellow.

iv. Salmonella confirmation

Nutrient Agar: Suspected colonies from identification were streaked on the surface of pre-dried nutrient agar plates and incubated at 37 ± 1°C for 24 ± 3 hours. This step allowed the isolated colonies to develop. Up to five suspected colonies per plate were purely cultured and then used for biochemical and serological confirmation.

Biochemical confirmation: After incubation on nutrient agar, the pure colonies were picked up and inoculated into Triple Sugar Iron agar (TSI; Merck KGaA, Germany), Motility Indole Lysine agar (MIL: Merck KGaA, Germany) and Urea broth (Merck KGaA, Germany). All inoculated biochemical media were incubated at 37 °C for 18 - 24 hours. After incubation, biochemical results were observed. In the case of *Salmonella*, the reaction would present the as following appearances of each media (Appendix 2.4).

Serological confirmation: All strains which were isolated and suspected as *Salmonella* were confirmed by serotype following the Kauffmann-White scheme. The serological confirmation of *Salmonella* antigens was done using slide agglutination testing with antisera from SIFIN (Germany). The summarized procedure on *Salmonella* serotyping is described in Appendix 2.5.

3.3.2.2 Salmonella enumeration

We used the 3 tube-Most probable number (MPN) technique to enumerate *Salmonella* spp. in samples by following ISO 21528-1:2004 (ISO, 2004). A series of 3 sequential MPN tubes was inoculated directly from the 1:10 dilution of original sample (in sample preparation step). Decimal dilution series 1:100 and 1:1,000 were made. Three sets of 3 tubes (10 ml from each dilution) were incubated for pre-enrichment. After incubation, *Salmonella* identification and confirmation steps were

following the same procedure as described above. This series of tubes represented dilutions from initial sample counted by gram or cm^2 or hand depended on kind of sample. The remainder of the sample homogenate was used for qualitative as above. The procedure of dilution for pre-enrichment in 3 tube-MPN method is described in diagram in Appendix 2.6. The number of *Salmonella* positive tubes in each dilution was used to calculate the number of *Salmonella* by using MPN tables (BAM, 1998).

3.3.3 Data collection from groups and stakeholders

Data and information were collected by using quantitative and qualitative tools to get information on potential risk factors of *Salmonella* contamination as well as perception and behavior of selected groups and stakeholders. Data were collected from the same study locations used for the biological sampling. While checklist and questionnaire were applied during biological sampling, focus group discussions (FGD) and in-depth interview (IDI) were done later between April and May 2013. An overview on questionnaires, checklists, IDI and FGD including targeted groups are presented in Table 5.

Table 2 Number of interviews, discussion groups, checklists and questionnaires

Tools/ groups/stakeholders/places	No. groups/districts	Total participant/interviewee/checklist
Quantitative approach		
Questionnaire		
Pig origin questionnaire (slaughterhouse owner)	3	12
Observation (checklist)		
Pig slaughterhouse	3	3
Pork shop	3	19
Qualitative approach		
Focus group discussion		
Slaughter worker	2	10
Pork seller	3	15
In-depth interview		
People living around slaughterhouse	3	9
Pork consumer	3	9
Public health staff	3	3
Veterinary staff	3	3

Questionnaire and checklists: We interviewed owners of 3 slaughterhouses (one in each district) using a questionnaire (Appendix 3) to gather information on slaughtered pigs. Questionnaires were used repeatedly at each slaughterhouse visit for biological sampling (4 consecutive visits) as explained in the sample collection section above. To observe presence or absence of objects or practices, we used checklists (Appendix 4) at these slaughterhouses and also at pork shops for sampling. Questionnaire and checklists were designed based on pre-observations on facilities in slaughterhouses and common hazards that might lead to risks of *Salmonella* contamination.

Focus group discussions: Following a developed outline in focus group discussions (see Appendix 5) focused on food safety in general targeting slaughter workers and pork sellers which were selected from the accessed slaughterhouses and markets. Focus group discussions were organized by calling a group of 5-6 participants and lead by one facilitator and a note taker (assist researcher). Additionally, audio recording was used following gained permission from all participants. Each discussion lasted about 1 - 1.5 hours.

Slaughterhouse workers groups: Two FGD (5 workers each) were done in Van Giang and Van Lam district. Participants worked in the slaughterhouse as permanent employees.

Pork sellers groups: A total of 3 sellers discussion groups were carried out, one in each of three sampled districts. Age of participants ranged from 31 to 55 years old. There had three kind of selling: retail only, both whole sale and retail, and wholesale only. Selling time is mostly in the morning (Appendix 6.1). All of participants used motorbike to transport their pork or carcass to the market.

In-depth interview: As shown in Table 5, in-depth interviews were done in public health and veterinary staffs, consumers and people living around the slaughterhouse. One veterinary and public health staff was selected in each of the three districts, one public health staff took care of food safety at commune level. Consumers (3 in each district) were mainly selected by asking sellers to introduce their potential consumers. Other consumer group interviewees were selected directly after seeing them buying pork at the market and willing to participate. To interview people living around the slaughterhouse, we randomly chose 3 households that were located within approximately 200 meters diameters to slaughterhouse and also willing to participate (only adult household members). The interview was operated by face-to-face interview. Interviewer used the outline prepared for each group. During interviews, taking notes and/or recording was done by interviewer or assistant with duration of about 20 to 45 minutes.

3.5 Data analysis

All data collected from slaughterhouses, markets and laboratory results were processed and recorded using Microsoft Excel 2007. Statistically significant computing was considered at *p-value* of 0.05.

The presence or absence and number of *Salmonella* were described using percentage. Checklist and questionnaire were used for statistical analyses to test related risk factors of *Salmonella* contamination. Odds ratio (OR), Chi square, Fisher' exact tests were applied to determine significant factors leading to *Salmonella* positive results. Results were used to consider significant differences among accessed slaughterhouses or markets.

Descriptive statistic (if applicable), transcript and content analysis were used to describe perception and practice behavior of people and relevant stakeholders related to *Salmonella* contamination in pork production in particular and related to food safety in general.

R Studio version 2.15.1 (General Public License) and Win Episcope (version 2.0, 1998) software were used to compute data. Endnote X3 version 3.0 (Thomson Reuters) was used to insert references.