CHAPTER III

MATERIALS AND METHODS

3.1 Study scope

The study investigated microbiological hygienic conditions in two classifications of slaughterhouses (Accredited and Unaccredited) in the National Capital Region (NCR), Philippines from the period of January to April 2013. A survey on slaughterhouse operations and practices was conducted prior to sample collection to create a profile for each slaughter facility based on type, capacity and the slaughter process. Such data was used for further categorizing of each facility and should help in determining an appropriate sampling scheme.

The study covered sample collection and processing for microbiological investigation, specifically: Total Viable Count (TVC) and isolation and enumeration of *Enterobacteriaceae* and detection of *Salmonella* from environmental samples and carcass swabs, and detection of *E. coli* and coliforms in water.

3.2 Sampling

3.2.1 Sample collection

Collection of samples was conducted in two slaughterhouses, NMIS-accredited and LGU-licensed pig slaughter establishments, in the NCR, specifically in Valenzuela City. The participating establishments were an NMIS-Accredited slaughterhouse and an LGU-licensed slaughterhouse. NMIS-Accredited slaughterhouses are those with valid and up-to-date accreditation by the NMIS and classified as either AA or A. The rest are similar slaughter facilities that have not undergone accreditation by the NMIS but are licensed for commercial slaughtering by

local government authorities. Both establishments were visited five times within the study period.

Samples collected from each visit to a slaughterhouse were transported for processing and incubation to the National Meat Inspection Service (NMIS) Central Meat Laboratory located in Quezon City within 5 hours of collection.

3.2.2 Sample size and sampling strategy

The following sample collection strategy was employed in the study:

Each establishment was visited 5 times for the duration of the study, with each visit conducted at a different day of the week to avoid any bias on that aspect. One visit per week was conducted to allow sufficient time for sampling, processing and data recording. Table 3 shows all samples obtained for the duration of the sample collection period.

Table 3 Sample size and sampling strategy

Facility	Carcass samples	Environmental Samples							Water Sample	Number of visits
	C1 - C4	SV	DT	ET	F	KB	KS	H	W	
NMIS-Accredited	4	2	2	2	2	2	2	2	1	5
LGU-Licensed	4	2	2	2	2	2	2	2	1	5

3.2.3 Sampling techniques

The techniques used for sampling are in accordance to ISO 18593:2004(E) or the International Standard on Microbiology of food and animal feeding stuffs –

Horizontal methods for sampling for surfaces using contact plates and swabs (ISO, 2004a).

Wet-and-dry double swab technique

The wet-and-dry double swab technique is a widely used method for collection of environmental swab samples and is the preferred method (Swanenburg et al., 2001, Botteldoorn et al., 2003) in many studies involving carcass sampling. This technique was performed by taking out a cotton swab with a breakable stem from its sterile wrapping and immersing it into a 10ml test tube of Buffered Peptone Water (BPW). The swab was then streaked vigorously on the sampling surface covering an area of 10 x 10cm^2, all the while rotating the swab between the thumb and forefinger. The swab was then replaced into the test tube containing BPW, with the stem aseptically broken off. The swabbing procedure was again performed on the same surface but this time with a dry cotton swab.

Collection of water samples

Water samples were collected during all visits to each facility. Sampling was done by first allowing water to flow for around 1 minute through faucets, hoses or other equipment used in the facility, then collecting a liter (1L) of water into a sterile glass container.

Samples collected from each visit were brought to the NMIS Central Meat Laboratory for processing and incubation within 12 hours of collection.

3.2.4 Sampling sites

Environmental samples

Samples were obtained from among different sites in the slaughtering line during each visit. Categorization of the slaughter facilities through the initial survey

determined the following specific environmental sampling sites for both slaughterhouse classifications:

- Scalding vat
- Dehairing Table
- Eviscerating/Splitting Table
- Floor
- Butcher's Knife (for sticking, dehairing, evisceration)
- Cleaver (for splitting)
- Workers' hands

A swab sample was taken from each sampling site during each of the five visits, not more than 30 minutes prior to the start of slaughterhouse operations.

Carcass Samples

Carcass sampling was performed using a non-destructive method as prescribed in International Standard (ISO) 17604:2003, Microbiology of food and animal feeding – Carcass sampling for microbiological analysis. Samples were obtained from four different sampling sites (4 x 25cm^2 covering a total area of 100cm^2 per carcass): back, neck, ham and belly using the wet-and-dry double swab technique as described in detail under sampling techniques. Carcasses were swabbed after the final wash while hanging on the rail system prior to loading on the transport vehicle.

3.3 Microbiological procedures

3.3.1 Decimal dilution of test samples

Samples obtained using the wet-and-dry double swab technique were prepared by first shaking thoroughly after which 1ml of the suspension was added to a test tube containing 9ml of Maximum Recovery Diluent (MRD; Merck KGaA, Germany) with 0.0075g agar-agar. The dilution was repeated until the desired number of dilutions

was obtained. In this study, all swab samples were diluted to up 10^{-6} and were used for both Total Viable Count and Isolation and Enumeration of *Enterobacteriaceae*.

3.3.2 Total Viable Count

The procedure that was followed to perform a Total Viable Count (TVC) was the horizontal method for enumeration of organisms – colony-count technique at 30°C also known as International Standard 4833:2003(E) (ISO, 2003) and using the Drop-plate Technique.

The Drop Plate Technique

Two separate 0.05ml of the suspension for each dilution were transferred to two Plate Count Agar (PCA; Merck KGaA, Germany) plates, each divided into six cells representing all dilutions. Drops were allowed to dry and solidify before PCA plates were incubated inverted at 30 °C ± 1 °C for 72 ± 3 hours. After the specified incubation period, manual counting of all colonies was performed.

3.3.3 Isolation and enumeration of *Enterobacteriaceae*

Isolation and Enumeration of *Enterobacteriaceae* from collected samples was based on the International Standard for horizontal methods for detection and enumeration of *Enterobacteriaceae* or ISO 21528-2:2004 (ISO, 2004b).

Inoculation and incubation

Inoculation of plates for this procedure was done in tandem with TVC, using the same series of decimal dilutions and employing the Drop-plate Technique. Two separate 0.05ml of the suspension for each dilution were dropped into two Violet Red Bile Dextrose (VRBD; Merck KGaA, Germany) Agar plates, each divided into six cells representing all dilutions. Drops were then allowed to dry and solidify before VRBD plates were incubated inverted at 37 °C for 24hours ±2hours.

Counting of colonies and selection for sub-culture

After incubation, each plate was examined for growth of colonies that met the typical characteristics of *Enterobacteriaceae* (pink to red or purple colonies with or without precipitation haloes). All cells with 30 or less characteristic colonies were counted and recorded and further classified on specific characteristics pending biochemical confirmation.

Sub-culturing for biochemical tests was done by random selection of five characteristic colonies for each classification. Selected colonies were inoculated on nutrient agar plates and incubated at 37 ^{o}C for 24hours ±2hours.

Biochemical tests for confirmation

Well-isolated characteristic colonies were selected from each subculture for the performance of the following biochemical tests for confirmation:

Oxidase test

A part of each well-isolated colony was taken using a wire loop, streaked on filter paper moistened by oxidase reagent and allowed to stand for around 10 seconds. A negative result was noted if within that period a dark blue color did not appear.

Fermentation test

Samples that tested negative for the oxidase test were subjected to the fermentation test. This was conducted by stabbing colonies into two glucose agar tubes using an inoculating needle. A layer of liquid paraffin was added to one of the tubes to create anaerobic conditions. Both tubes were incubated at 37 ^{o}C for 24hours ± 2hours.

A development in the color of the medium from purple to yellow after incubation implied a reduction in pH and was thus interpreted as a positive result. The confirmation of selected colonies as *Enterobacteriaceae* using both biochemical tests should yield Oxidase-negative and Glucose-positive results. Only colonies yielding such results were included in the final count of *Enterobacteriaceae* for each dilution.

3.3.4 Detection of *E. coli* and coliforms

The method that was employed in detection of *E. coli* and coliforms in water samples was the ten tube Most Probable Number (MPN) coliform test in accordance to International Standard (ISO) 9308-2:1990(E), Water quality – Detection and enumeration of coliform organisms, thermotolerant coliform organismsand presumptive *Escherichia coli* (ISO, 1990).

Water used in the slaughter line was collected (1L) from the tap by letting water flow for about 1 minute. Testing was done as follows:

- 10 ml of undiluted water sample was inoculated into 10 testtubes each containing 10ml Double-strength Lauryl Sulfate Tryptose Broth (D-LSB; Merck KGaA, Germany) medium
- 1 ml of undiluted water sample was inoculated into 10 testtubes each containing 10 ml Single-strength Lauryl Sulfate Tryptose Broth (S-LSB; Merck KGaA, Germany) medium
- 0.1 ml of undiluted water sample was inoculated into 10 testtubes each containing 10ml Single-strength Lauryl Sulfate Tryptose Broth (S-LSB; Merck KGaA, Germany) medium

The tubes were incubated at 37°C and examined after 24 to 48 hours ± 2hours for growth and gas formation which may be observed in a Durham tube or in its absence, effervescence when tubes are gently agitated.

All tubes indicative of a positive result were subjected to confirmatory tests as follows: Each positive LSB tube was gently agitated after which one or more loopfuls of suspension was transferred using a sterile inoculating loop to a tube of Brilliant Green broth (BG; Merck KGaA, Germany) for coliforms and EC (Merck KGaA, Germany) and Tryptone Water (TW; Merck KGaA, Germany) for *E. coli*.

BG tubes were incubated for 48 ± 2 h at 37°C while the EC and TW tubes were incubated at 44°C. Tubes were examined for gas production and reaction to addition of Kovac's reagent (Merck KGaA, Germany) to TW tubes and results were recorded. MPN was calculated using 10 tube MPN Tables.

3.3.5 Detection of *Salmonella*

The methodology that was utilized in the detection of *Salmonella* in obtained samples was based on the International Standard on horizontal method for detection of *Salmonella* or ISO 6579:2002(E) (ISO, 2002) and was performed as follows:

Pre-enrichment

Swab samples were mixed with 100 ml Buffered Peptone Water and incubated at 37ºC for 18 hours ± 2 hours. This initial step stimulates growth of all organisms and allows resuscitation of those stressed during the sampling process.

Selective enrichment

The broth from pre-enrichment was first shaken before transferring 0.1ml to 10ml pre-warmed Rappaport-Vassiliadis (RV) Enrichment Broth (Merck KGaA, Germany). This was then incubated at 42 ºC for 24 hours ± 3hours.

Secondary selective enrichment was also done by transferring 1ml of pre-enrichment broth to 10 ml Mueller-Kauffmann Tetrathionate/Novobiocin Broth

(MKTTn) broth (Merck KGaA, Germany) which was then incubated at 37 ºC for 24 hours ± 3hours.

Isolation and identification

After the prescribed incubation period, the selective media broth is then streaked separately on the selective media Brilliant Green Agar (BGA; Becton, Dickinson and Company, USA) Modified and Xylose Lysine Tergitol-4 (XLT-4; Merck KGaA, Germany) Agar plates which will be incubated for 24hours ± 3hours at 37ºC ± 1ºC. After incubation, the plates will be examined for growth of colonies with the typical specific characteristics of *Salmonella* on each type of agar: BGA – pink colored colonies surrounded by a red zone; XLT – black-centered red colonies. Up to 5 colonies per plate are then selected and streaked on the surface of nutrient agar plates and allowed to incubate at 24hours ± 3hours at 37ºC ± 1ºC

Biochemical confirmation

Colonies that would grow in the nutrient agar will be inoculated into a Triple Sugar Iron (TSI; Merck KGaA, Germany), Motility Indole Lysine (MIL; Merck KGaA, Germany) and Urea (Becton, Dickinson and Company, USA) slants. These will be incubated at 37ºC for 18 - 24 hours. Results after incubation will be interpreted as stipulated in ISO 6579:2002(E).

Confirmation of Salmonella strains

Colonies confirmed through biochemical tests were submitted for serotype confirmation by agglutination to the WHO National Salmonella and Shigella Center (NSSC), National Institute of Health of Thailand, Department of Medical Sciences, Ministry of Public Health, Thailand.

3.4 Calculation of TVC and *Enterobacteriaceae* counts

Swab samples from in this study were prepared using a decimal dilution series where TVC and *Enterobacteriaceae* counts were calculated using the following formulae:

Formula for environmental samples

$$N = \frac{\Sigma C}{[(1*n_1) + (1*n_2)]*d*2.5}$$

Formula for carcass samples

$$N = \frac{\Sigma C}{[(1*n_1) + (1*n_2)]*d*1}$$

Where:

N = number of colonies; N was subsequently converted into log 10.

ΣC = total number of colonies counted

n1 = number of colonies in the first dilution

n2 = number of colonies in the second dilution

d = decimal dilution from which first count was obtained