CHAPTER III

MATERIALS AND METHODS

3.1 Study scope

The study covered the determination of prevalence and identification of *Campylobacter* spp. on breast skin of fresh chicken meat at wet local retail markets in Nueva Ecija, Philippines by using microbiological culture method, PCR method and biochemical test. Moreover, it also determined the quantitative load of *Campylobacter* by using semi-quantitative detection method and determined antimicrobial resistance patterns of the *Campylobacter* isolates.

3.2 Study type

The study was a cross-sectional study which was mainly concerned in determining the prevalence of *Campylobacter* contamination, determining quantitative load of the pathogen and antimicrobial resistance patterns of *Campylobacter* isolates in local wet markets in Nueva Ecija, Philippines.

3.3 Study site and period

The study was conducted at College of Veterinary Science and Medicine, Central Luzon State University, Science city of Munoz, Nueva Ecija, Philippines during the period of January to April 2013.

3.4 Sample collection

Fresh breast meat of chicken with skin was bought from city markets, from local wet markets, in the province of Nueva Ecija mainly from three cities which were Cabanatuan city, Gapan city and San Jose city and one municipality which was Guimba. These areas represented each district in the province being the largest ones and having the biggest local wet markets. Samples were collected from stalls selling chicken meat using random sampling. Each sample collected was placed in a sterile plastic bag to avoid cross-contamination and was transported to the laboratory where skin sampling was aseptically undertaken. Fifteen samples were processed for each week. The samples collected were kept at +2 to 8 °C and free of external contamination during transportation to the laboratory. All samples ideally reached the laboratory within 4 hours of sampling. These sample collection procedures were patterned from the EU COMMISSION DECISION (2007/516/EC) Part D on specimen collection and handling of *Campylobacter* spp. in broiler carcass.

3.5 Sample size calculation

Sample size was calculated using the Win Episcope program version 2.0 for a 50% expected prevalence with a 95% confidence level and a 10% accepted error. The resulting sample size was 97 samples. However, sample size was increase to 120 samples as suggested and agreed by advisors and panel members.

3.6 Semi-quantitative detection method of *Campylobacter* spp.

The semi-quantitative detection method of *Campylobacter* spp. was done in accordance to ISO 10272-3:2010 (E) Part 3 "Microbiology of food and animal feeding stuffs-Horizontal method for detection and enumeration of *Campylobacter* spp."(ISO, 2010).

Using a sterile instrument and aseptic technique, the breast skin was removed by avoiding any fat to a 15 g test portion which was placed into a stomacher bag. One

hundred twenty milliliter of prepared Bolton broth (Oxoid, UK) with supplement (Oxoid, UK) and 5% sterile laked horse blood was introduced to the sample in the stomacher bag and blended for 1-2 minutes to homogenize. Ninety milliliter from the initial suspension was transferred to 100 ml bottle. This corresponded to 10g of the test portion which corresponded to 10^1. Ten ml of the initial suspension was transferred to a culture tube. This corresponded to 10^0. A 10-fold dilution series up to 10^{-4} from 10^0 dilutions were prepared by transferring 1 ml to tubes containing 9ml Bolton broth. A quantity of 1 ml from the highest dilution was discarded. The dilutions corresponded to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}.

Sample preparation, initial suspension and dilutions. All Bolton broths were incubated for 48 hours at 42 ˚C in microaerobic conditions. For each enriched Bolton broths, one loop (approximately 10 µl.) was streak on modified charcoal-cefoperazonedeoxycholate-agar (mCCDA) (Oxoid, UK) with supplement (Oxoid, UK), which was divided into four quarters, on their corresponding quarter and the plates were then incubated at 42r °C for 44 to 48 hours in microaerobic conditions. *Campylobacter* enumeration was done in all samples. The plates were examined for typical or suspect colonies of *Campylobacter* spp. The typical colonies were grayish on mCCD agar, often with a metallic sheen, and were flat and moist, with a tendency to spread.

Confirmation and interpretation. For confirmation, from each plate at least one colony considered to be typical or suspected as being *Campylobacter* spp. was taken. Each of the selected colonies was streak on prepared Columbia blood agar (Oxoid, UK) plate in order to allow the development of well isolated colonies. Then incubated at 42 °C for 44 to 48 hours in microaerobic conditions. Pure cultures were examined on a glass slide with cover slip using a microscope for morphology and motility. For the detection of oxidase, a portion of a well-isolated colony was taken from each individual Columbia blood agar plate and streak it on filter paper moistened with the oxidase reagent (Becton, Dickinson and Company, US). The appearance of a mauve, violet or deep blue color within 10 seconds indicated a positive reaction.

The results were interpreted to identify *Campylobacter* spp. by biochemical characteristics as presented on a table below.

Table 2 Characteristics of *Campylobacter* spp. (source: ISO/TS 10272-3, 2006)

Tests	**Characteristics**
Morphology	small curve bacilli
Motility	Characteristics
Oxidase	+

+=positive

Table 3 Reading of growth results of confirmed *Campylobacter* (source: ISO/TS 10272-3, 2011)

Quantity(g)	**Growth of Confirmed *Campylobacter* spp.**						
10^1	-	+	+	+	+	+	+
10^0	-	-	+	+	+	+	+
10^{-1}	-	-	-	+	+	+	+
10^{-2}	-	-	-	-	+	+	+
10^{-3}	-	-	-	-	-	+	+
10^{-4}	-	-	-	-	-	-	+
MPN/g	0	0.23	2.3	23	230	2400	∞
L_{CL}	0	0.019	0.19	1.9	19	190	580
U_{CL}	0.33	2.7	27	270	2,700	300	∞

If all test are negative, the result should be reported as MPN=0/g (upper confidence limit, T_1 0,33/g); if all test are positive, the results should be reported as MPN=∞ (lower confidence limit T_0 580/g).

3.7 *Campylobacter* spp. confirmation by molecular technique

DNA extraction by boiling method. One to two well isolated colonies was taken from Columbia blood agar using a sterile loop and mixed with 1ml sterile distilled water. Then centrifuge for 4 minutes at maximum speed. Pellet was then washed in 200µl sterile distilled water. Pellet was then re-suspended in 50µl sterile distilled water then boiled for 10 minutes. After that, tubes were placed on ice for 10 minutes then centrifuged for 5 minutes at maximum speed. And supernatant stored at 4°C until used in further analysis.

Multiplex PCR for verification and identification of Campylobacter spp. A multiplex PCR assay was used to simultaneously identify genes from the two most important clinically relevant *Campylobacter* spp. Those genes selected were *hipO* from *C. jejuni*, *glyA* from *C. coli*. and the internal control 23S rRNA (Wang et al., 2002).

Table 4 Primer sequences used in the multiplex PCR assay and the expected sizes of the products (source: Wang et al., 2012)

Primer	Amplicon size (in bp)	Sequence (5´-3´)	Target gene	Gene location (bp)
CJF	323	ACTTCTTTATTGCTTGCTGC	*hipO*	1662-1681
CJR		GCCACAACAAGTAAAGAAGC		1984-1965
CCF	126	GTAAAACCAAAGCTTATCGTG	*glyA*	337-357
CCR		TCCAGCAATGTGTGCAATG		462-444
23SF	650	TATACCGGTAAGGAGTGCTGGAG	23S rRNA	3807-3829
23SF		ATCAATTAACCTTCGAGCACCG		4456-4435

Each multiplex PCR tube contain 1.5 µl deoxynucleoside triphosphate [10mM] (iNtRon), 2.5 µl of 10x reaction buffer [2.5mM] (20 mM Tris-HCl; 10 mM $[NH_4]2SO_4$; 10 mM KCl; 2 mM Mg SO_4; 0.1% Triton X-100 pH 8.8 @ 25°C) (Promega, USA), 8 µl $MgCl_2$ [25 mM] (Promega, USA); 0.25 µl of each *C. jejuni*; 0.5 µl of each *C. coli* and 0.2 µl 23S rRNA primer , 0.1 µl GoTaq Flexi [5U/ul] (Promega, USA), and 2 µl of template DNA. The volume was adjusted with sterile distilled water and gave 25 µl. DNA amplification was carried out in a thermal cycler (C1000

Touch, BIO-RAD) using an initial denaturation step at 95°C for 4 min then followed by 30 cycles of amplification at 94°C for 30 sec, annealing at 60°C for 45 sec, and extension at 72°C for 45 sec, ending with a final extension at 72°C for 5 min. As positive controls, PCR products with the *Campylobacter* reference strains *C.jejuni* NCTC 11168 and *C. coli* NCTC 11353 as templates were amplified. All PCR products were analyzed by gel electrophoresis through 2% agarose gel , stained with GelRed, in 1xTAE buffer and visualized by blue light led transilluminator (Wang et al., 2002).

3.8 Antimicrobial sensitivity testing

One strain from each *Campylobacter*-positive sample was selected and streaked out with sterile swabs on Mueller-Hinton agar (Becton, Dickinson and Company, US) supplemented with 5% defibrinated sheep blood for susceptibility tests according to the Clinical Laboratory Standards Institute. Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method. The following antimicrobial impregnated disks were used: ciprofloxacin (5µg), erythromycin (15 µg), tetracycline (30µg), ampicillin (10 µg) (all from Oxoid, UK) and gentamicin (10 µg) from (MAST Diagnostics, UK). After incubation at 42°C for 48 h in a microaerophilic atmosphere, the susceptibility of the *Campylobacter* spp. to each antimicrobial agent was measured and the results was interpreted in accordance with interpretative criteria provided by CLSI (2010). The table below was used to determine the antimicrobial susceptibility of each drug by measuring the zone diameter.

Table 5 Breakpoints of disk diffusion method used to determine antimicrobial susceptibility of *Campylobacter* isolates (source: CLSI, 2010)

Antimicrobial agent	Disk conc. (µg)	Zone diameter Breakpoint (mm)		
		S	I	R
Ampicillin	10	≥17	14-16	≤13
Tetracycline	30	≥15	12-14	≤11
Gentamicin	10	≥15	13-14	≤12
Erythromycin	15	≥23	14-22	≤13
Ciprofloxacin	5	≥21	16-20	≤15

(S=susceptible; I=intermediate; R=resistant)