CHAPTER 3

MATERIALS AND METHODS

This study consisted of 3 parts; primary isolation and identification, differentiation of *Campylobacter spp.*, and antimicobial resistance testing.

Part A: Primary isolation and identification

This part of the study is described in sections 3.1 to 3.2. After the samples were collected during May to July in 2000 – 2002, the primary isolation and identification of these samples were conducted each year by the Minority International Research Training Project at the Central Laboratory of the Faculty of Veterinary Medicine, Chiang Mai University. The isolates were frozen and used part B of these studies.

Part B: Differentiation of *Campylobacter spp.*

This part of the study is described in sections 3.3 to 3.4. The experiment of this thesis was performed in 2003. The species of *Campylobacter* were identified by Multiplex Polymerase Chain Reaction at the Central Laboratory of the Faculty of Veterinary Medicine, Chiang Mai University.

Part C: In vitro susceptibility testing

This part of the study is described in section 3.5. The studies were done by the Minority International Research Training Project at the Central Laboratory of the Faculty of Veterinary Medicine, Chiang Mai University.

3.1 Sample collection

These samples were collected during May to July of each of the three years 2000, 2001, and 2002. The samples were collected from six chicken farms, one slaughterhouse and two meat venders at the fresh market in Chiang Mai area. (Table 3.1)

3.1.1 At the chicken farms

Six chicken farms from two companies, that raised chickens until they reached the market age (about 45 to 55 days old) participated in this study. All these farms were included as they met the criteria for in conclusion: having chickens of appropriate age at the first sampling time (approximately 40 days) and being located within 80 kilometers radius from the laboratory. Twenty-five chickens were randomly selected approximately one week before they were sent to slaughter. Fecal swabs from the cloaca were collected by using sterile cotton swabs stored in Stuart's transport media. (RCM supply, Bangkok, Thailand).

Chicken farm workers and their non-farm neighbors (as controls) were asked to submit 10 grams of stools in sterile plastic cups containing Cary-Blair medium. Swabs of pen floors and feed trays were also collected using sterile gauze pads soaked with 10 ml of sterile skim milk.

3.1.2 At the slaughterhouse

The carcass swabs were collected after killing and defeathuring of the chicken but before putting into the chilling tank. Sterile cotton swabs were used to collect fecal swabs in Stuart's media transport and the sterile 25cm^2 gauze pads were used to collect the samples from the area under both wings. Both carcass swabs were put in the plastic bags with 10 ml of sterile skim milk for transportation.

3.1.3 At the fresh market

Chicken meat (a thigh from each chicken) were purchased from two meat venders at the fresh market.

All samples from 3.1.1. to 3.1.3 were stored on ice during transportation to the laboratory and processed within 12 hours after collection.

3.2 Primary isolation and Identification

All of the fecal swabs, carcass swabs, stool samples from farm workers, and environmental samples were inoculated directly onto the selective media, Karmali agar or Preston agar. The plates were incubated under microaerobic condition (5%O_2, 10%CO_2) in plastic bags at 42°C for up to 48 hours. The samples from chicken meat at the fresh market, however, were inoculated in Bolton broth as an enrichment media for 48 hours to resuscitate potentially damaged cells before inoculation on selective media (KSA or Preston agar) under the same condition.

After 48 hours, colonies characteristics of *Campylobacter* were examined. Suspected colonies were confirmed by oxidase test (Dryslide, BBL), catalase test (3%H_2O_2) and gram stain. *Campylobacter spp.* was identified by the gram-negative spiral rods with both positive oxidase and catalase test. The isolates were frozen and stored in 30%glycerol with Mueller-Hinton broth at –70°C.

3.3 Recover of stock isolates

The stock isolates were inoculated onto Brucella agar supplemented with 5% defribrinated sheep blood and incubated at 42°C for 48 hours under microaerobic conditions. After the second passage on the Karmali agar, colonies morphology were inspected and gram staining was performed for the purpose of confirmation the recovered samples. Then the recovered samples were processed further to identify the species as described in 3.4.

3.4 Differentiation of *Campylobacter species*

3.4.1 DNA template preparation

3.4.1.1 Whole cell procedure

DNA was prepared by the whole-cell procedure. The concentration of culture was adjusted to 0.5 McFarland (10^8 cfu/ml) in Bolton broth by using a colorimeter. One ml of culture was transferred in the 1.5 ml Eppendorf tube and heated at 100°C for 10 minutes. Templates were kept in 4°C until processing as reported by Wang et al.(2000).

3.4.1.2 Phenol - Chloroform DNA extraction

100 ul of culture in Bolton broth were put onto 500 µl of D-solution in 1.5 ml-Eppendorf tube. Equal volume of saturated Phenol and chloroform 500 µl were added to the solution. After gently mixing, the solution was then centrifuged at 13,000 rpm for 5 minutes. 500 µl of the clear supernatant were transffered to the new sterile Eppendorf tube. DNA was precipitated by adding 1000 µl of absolute ethanol and leave at –70°C for 30 minutes. After the solutions were centrifuged at 13,000 rpm for 10 minutes, the DNA solution was washed by 1000 µl of 70% ethanol for 2 times. The DNA precipitate was dried after which 100 µl of TE buffer were added. This DNA solution was stored at –20°C and thawed at 4°C before using.

3.4.2 Primers

Six pairs of primers were used to identify the genes *hipO* from *C.jejuni*, *glyA* from *C.coli, C.lari* and *C.upsaliensis*, *sapB2* from *C.fetus subsp. Fetus;* the internal control 23S rRNA. The primer sequences are showed in table 3.2.

3.4.3 Multiplex PCR condition

Each multiplex PCR tube contained 200 μM deoxynucleoside triphosphate; 2.5 μl of 10X reaction buffer (500mM Tris-HCl[pH7.3]), 100 mM KCl, and 50 mM ($[NH_4]_2SO_4$); 20 mM MgCl2; 0.5 μM *C.jejuni* and *C.lari* primers; 1μM *C.coli* and *C.fetus* primers, 2 μM *C.upsaliensis* primers; 0.2 μM 23S rRNA primer (from Table3.2);1.25 U of *Tag* DNA polymerase, and 2.5 μl of whole cell template DNA. The volume was adjusted with sterile distilled water to give 25 μl. DNA amplification was carried out in a thermocycle using an initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), ending with a final extension at 73°C for 7 min.(Wang, et al.,2002) PCR reaction were carried out in Thermohybate thermocycler (Biorad, NY).

3.4.4 Reading of Results

The PCR-amplified products pattern of samples were compared with the *Campylobacter* reference strains as templates following 1% agarose gel electrophoresis at 5 V/cm in 1X TA or TBE buffer using Minicell electrophoresis (Biorad, NY). The Gel was stained with 0.05 mg/l $EtBr_2$. The resulting band pattern was visualized and recorded using Geldoc2000 (Biorad, NY)

3.5 In vitro susceptibility testing

In vitro susceptibility testing was done using the microbroth dilution method, following guidelines provided by the US National Committee on Clinical Laboratory Standards (NCCLS,1997). Bacterial isolates from frozen stock were grown on Brucella agar supplemented with 5% defribrinated sheep blood (BASB) for 48 hours at 37°C under microaerobic conditions (85%N_2, 5%CO_2, 10%O_2). Individual colonies from each plate were subcultured on BASB under similar growth conditions. Bacteria were scraped from the BASB with a sterile cotton swab and suspended in 5 ml H_2O. The turbidity was adjust to a 0.5 McFarland standard using a standard solution, and one ml of the bacterial suspension was then added to 9 ml of Haemophillus testing medium (HTM). The final concentration of the inoculum was approximately 8 x 10^5 CFU/ml.

Customized SensiTitre plates were purchased pre-made from TREK diagnostic Systems, Inc. with ampicillin, ceftiofur, cephalothin, clindamycin, erythromycin, florfenicol, nalidixic acid, streptomycin, tetracycline, trimethoprim-sulfamethoxazole. These antimicrobials were chosen on the basis of their importance in treating human *Campylobacter* infections and to provide diversity in representation of different antimicrobial classes. Antimicrobial concentrations on the plates ranged from 0.03 to 256 μg/ml, depending on the antimicrobial agent. *C.jejuni* ATC33560 was used as a quality control strain since it was shown to produce repeatable antimicrobial resistance profiles in microbroth dilution testing . Each plate was inoculated by adding 100 μl of the bacterial suspension to the plate using an

autoinoculator. Plates were covered with a gas permeable seal and incubated at 42°C at 5%CO_2 for 48 hours.

The minimal inhibitory concentration (MIC), the lowest concentration of an antimicrobial agent that inhibits growth of the bacteria, was recorded manually. Wells with turbidity or an accumulation of bacteria at the bottom were considered positive for bacterial growth. The breakpoint used to categorize isolates as resistant or not resistant for each antimicrobial agent for *Campylobacter spp.* were those recommended by the US National Antimicrobial Resistance Monitoring System (NARMS).

3.6 Statistical analysis

Chi-square test or Fisher exact test was used to compare the proportion of each species of *Campylobacter* isolated from different sources and different sample types.

Table 3.1 Number of samples tested.

Source	Sample type	2000	2001	2002	Total
Farm	Cloacal swab	97	126	41	264
	Environment	-	1	14	15
	Worker	-	2	3	5
Slaughterhouse	Cloacal swab	42	25	-	67
	Carcass swab	4	28	-	32
Market	Meat	-	32	-	32
	Total	143	214	58	415

Table 3.2 Primer sequences used in the multiplex PCR assay and the expected sizes of the products

Primer	Size (bp)	Sequence(5'-3')	GenBank Accession No.	Target gene	Gene location (bp)
CJF	323	ACT TCT TTA TTG CTT GCT GC	Z3690	*hipO*	1662-1681
CJR		GCC ACA ACA AGT AAA GAA GC			1984-1965
CCF	126	GTA AAA CCA AAG CTT ATC GTG	AF136494	*glyA*	337-357
CCR		TCC AGC AA TGT GTG CAA TG			462-444
CLF	251	TAG AGA GAT AGC AAA AGA GA	AF136495	*glyA*	318-337
CLR		TAC ACA TAA TAA TCC CAC CC			568-549
CUF	204	AAT TGA AAC TCT TGC TAT CC	AF136496	*glyA*	63-82
CUR		TCA TAC ATT TTA CCC GAG CT			266-247
CFF	435	GCA AAT ATA AAT GTA AGC GGA GAG	AF048699	*sapB2*	2509-2532
CFR		TGC AGC GGC CCC ACC TAT			2943-2926
23SF	650	TAT ACC GGT AAG GAG TGC TGG AG	Z29326	*23S rRNA*	3807-3829
23SR		ATC AAT TAA CCT TCG AGC ACC G			4456-4435

Table 3.3 Minimal Inhibitory Concentration (MIC) dilution ranges and breakpoint values for determination of antimicrobial resistance for *Campylobacter*, based on the US National Committee on Clinical Laboratory Standard (NCCLS) recommendations.

Antimicrobial agents	Code	Antimicrobial agents Concentration Range (μg/ml)	Resistance Breakpoint (μg/ml)
ampicillin	AMP	2-64	≥ 32
ceftiofur	CEF	0.5-16	≥ 8
cephalothin	CEP	1-32	≥ 32
ciprofloxacin	CIP	0.016-32	≥ 4
clindamycin	CLI	0.032-256	≥ 4
erythromycin	ERY	0.047-256	≥ 8
florfenicol	FLO	2-16	≥ 16
nalidixic Acid	NA	0.047-256	≥ 32
streptomycin	STR	32-356	≥ 64
tetracycline	TET	0.023-32	≥ 16
trimethoprim-sulfamethoxazole	COT	012/2.4 - 4/76	≥ 4/76