

MOLECULAR CHARACTERIZATION OF *CAMPYLOBACTER* ISOLATED FROM CHICKENS AND HUMANS IN NORTHERN THAILAND

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Abstract. A study was conducted in northern Thailand to characterize *Campylobacter* spp isolated from chickens at farms, slaughterhouse, and chicken meat at fresh markets, and stools from chicken farmers as well as neighboring crop farmers who served as controls. The *Campylobacter* isolates were collected during the rainy months of the years 2000, 2001, and 2002. Standard methods were used for primary isolation and identification of *Campylobacter* and the resulting isolates were frozen and stored in 30% glycerol with Mueller-Hinton broth at -70°C until used in 2003. A multiplex PCR assay was used for differentiation of the *Campylobacter* spp. A total of 415 *Campylobacter* spp were isolated from 849 (48.9%) samples from chickens at the farm, slaughter house and fresh chicken meat market. *Campylobacter* spp were isolated from 5 of the 129 (3.9%) chicken farmers but none from the 100 neighboring crop farmers. *C. jejuni* was the most prevalent (42.5%) at the farm, followed by *C. coli* (39.1%) and other species (8.0%). In contrast, *C. coli* was the most prevalent at the slaughter house (72.4%) while *C. jejuni* was only 17.2% and others 3.4%. Similarly, at the fresh chicken market, *C. coli* was the most prevalent (54.4%) while *C. jejuni* was 26.5% and others were 13.2%. *Campylobacter* spp isolated from the chicken farmers were predominantly (75%) *C. coli* and the rest (25%) were *C. jejuni*. The results of the study show that both *C. coli* and *C. jejuni* are highly prevalent in chickens, along the chicken production system and in chicken farmers in northern Thailand. Critical control points for exposure and contamination of the chicken meat supply should be identified so that methods can be developed to protect human exposure to *Campylobacter* spp.

INTRODUCTION

Campylobacter infection in humans results in an acute gastroenteritis illness, which causes diarrhea, fever, and abdominal cramps (Allos, 2001). However, *Campylobacter* spp is one of the 4 most importance food-borne bacteria that causes 3 times higher mortality than the background population (Helms *et al*, 2003).

Campylobacter infection is often a pediatric disease in developing countries. Although many developing countries do not have national surveillance programs for *Campylobacter* infection, case-control community- based studies have provided the incidence estimates of *Campylobacter* infec-

tion in children under 5 years old to be 40,000 to 60,000 cases per 100,000 population in developing countries and 300 cases per 100,000 population in developed countries. The incidence rates in the general population, however, are approximately 90 cases per 100,000 population in either developed or developing countries. The isolation and incidence rates of *Campylobacter* infection in some developing countries have increased recently, which has often been attributed to improved diagnostic methods (Cocker *et al*, 2002).

The consumption of chicken was identified as an important risk factor for *Campylobacter* infection in sporadic cases, while consumption of milk was usually found to be the cause of outbreaks of infection (Adegbola *et al*, 1990; Michino and Otsuki, 2000; Chattopadhyay *et al*, 2001). However, the evidence from a prospective study did not show a temporal association between contamination of chickens and human Campylobacteriosis,

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particularly during seasonal peaks, which suggested that many cases did not originate from chickens (Wilson, 2002). In Thailand, prevalence of *Campylobacter* in chickens was found to be 85% at the farm, 37% at the slaughterhouse and as high as 47% in chicken meat sold at fresh market (Padungtod *et al*, 2002). Comparable prevalence of *Campylobacter* has been reported in France in both chicken production (66.3%) (Denis *et al*, 2001) and raw retail chicken (57%) (Wilson, 2002).

During the past 3 years, the Faculty of Veterinary Medicine at Chiang Mai University, in cooperation with the Population Medicine Center at Michigan State University (USA), has been studying the epidemiology and antimicrobial resistance of *Campylobacter* isolated from chickens and chicken farm workers in northern Thailand. However, the species of those *Campylobacter* isolated were not identified, which would be essential in order to complete the picture of the risk that *Campylobacter* infections pose to the consumer by consumption of contaminated chicken meat. Therefore, this study was aimed at comparing species of *Campylobacter* isolated from chickens at the farms, slaughterhouse, and the market and from farm workers. The specific hypothesis tested was that the molecular *Campylobacter* types isolated from chickens and chicken meat are identical to those isolated from the chicken farm workers.

MATERIALS AND METHODS

Sample collection

The samples used in this study 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 vendors at the fresh market (Table 1).

Samples from chicken farm. Six chicken farms were included as they met the criteria for inclusion, namely having chickens age approximately 40 days at the first sampling time and being located within 80 km 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 neighboring crop farmers (as controls) were asked to submit 10 grams of stools in sterile plastic cups containing Cary-Blair medium (Paradisa). Swabs of pen floors and feed trays were also collected using sterile gauze pads soaked with 10 ml of sterile skim milk.

Samples from the slaughterhouse. Carcass swabs were collected after killing and defeathering of the chickens but before putting into the chilling tank. Sterile cotton swabs were used to collect fecal swabs in Stuart's medium transport and the sterile 25-cm² gauze pads were used to collect 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.

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

All samples from farm, slaughterhouse, and

Table 1
Number of isolates available.

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

market were stored on ice during transportation to the laboratory and processed within 12 hours after collection.

Primary isolation and identification

All swabs, carcass swabs, stool samples from farm workers, and environmental samples were inoculated directly onto the selective medium, Karmali agar or Preston agar. The plates were incubated under microaerobic conditions (5% O₂, 10% CO₂) 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 medium for 48 hours to resuscitate potentially damaged cells before inoculation on selective medium (KSA or Preston agar) under the same conditions.

After 48 hours, suspected colonies of *Campylobacter* were examined by oxidase test (Dryslide, BBL), catalase test (3% H₂O₂) and Gram stain. *Campylobacter* spp was identified by the gram-negative spiral rods with both positive oxidase and catalase test results. The isolates were frozen and stored in 30% glycerol with Mueller-Hinton broth at -70°C.

Recovery of stock isolates

The stock isolates were inoculated onto Brucella agar supplemented with 5% defibrinated 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 in order to confirm the recovered samples.

Differentiation of *Campylobacter* species

The differentiation of *Campylobacter* spp was accomplished using the multiplex PCR assay as reported by Wang (2002) as follows.

DNA template preparation

Whole cell procedure. The concentration of culture was adjusted to 0.5 McFarland (10⁸ cfu/ml) in Bolton broth by using a colorimeter. One ml of culture was transferred to 1.5 ml Eppendorf tube and heated at 100°C for 10 minutes. Templates were kept in 4°C until processing.

Phenol - chloroform DNA extraction. One hundred ml of culture in Bolton broth were put into 500 µl of D-solution in 1.5 ml Eppendorf tube. Equal volume (500 µl) of saturated phenol and

chloroform was added to the solution. After gently mixing, the solution was then centrifuged at 13,000 rpm for 5 minutes. Five hundred µl of the clear supernatant were transferred to a new sterile Eppendorf tube. DNA was precipitated by adding 1,000 µl of absolute ethanol and leaving at -70°C for 30 minutes. After the solution was centrifuged at 13,000 rpm for 10 minutes, the DNA solution was washed twice by 1,000 µl of 70% ethanol. 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 use.

Primers. Six pairs of primers were used to identify the gene *hipO* from *C. jejuni*, *glyA* from *C. coli*, *C. lari* and *C. upsaliensis*, and *sapB2* from *C. fetus* subsp *fetus*; the internal control was 23S rRNA.

Multiplex PCR assay. Each multiplex PCR tube contained 200 µM deoxynucleoside triphosphate; 2.5 µl of 10x reaction buffer [500 mM Tris-HCl (pH7.3), 100 mM KCl, and 50 mM (NH₄)₂SO₄]; 2 mM MgCl₂; 0.5 µM *C. jejuni* and *C. lari* primers; 1 µM *C. coli* and *C. fetus* primers, 2 µM *C. upsaliensis* primers; 0.2 µM 23S rRNA primer; 1.25 U of *Taq* 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 thermocycler using an initial denaturation step at 95°C for 6 minutes followed by 30 cycles of amplification (denaturation at 95°C for 0.5 minute, annealing at 59°C for 0.5 minute, and extension at 72°C for 0.5 minute), ending with a final extension at 73°C for 7 minutes. PCR reaction were carried out in Thermohybate thermocycler (Biorad, NY).

Reading of results. The PCR-amplified products patterns 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 EtBr₂. The resulting band pattern was visualized and recorded using Geldoc2000 (Biorad, NY).

Statistical analysis

Chi-square test or Fisher's exact test was

used to compare the proportion of each species of *Campylobacter* isolated from different sources and different sample types.

RESULTS

Recovered *Campylobacter* isolates

A total of 415 isolates of *Campylobacter* were identified from 849 collected samples and inoculated onto Brucella agar supplemented with 5% sheep blood. Only unrecovered isolates from the first attempt were resuscitated in Bolton broth before inoculating onto the same medium. After second passage, these cultures were confirmed with colony morphology and Gram stain. Finally, 271 of the 415 (65.3%) of isolates were recovered (Table 2).

Optimization of the assay

The reference strain of *Campylobacter jejuni* 33560 was used for optimization of the assay. Two methods of DNA template preparations were compared; whole cell procedure and phenol-chloroform extraction. Both methods produced the amplified products, however, the whole cell procedure gave better results in the initial trial.

Magnesium chloride concentration used varied from 1, 1.5, 2, 2.5, 3, 3.5 mM. However, 2 mM magnesium chloride was used because this concentration gave the best amplification efficiency. The concentration of DNA templates was also optimized. The concentration of 1×10^8 , 1×10^9 , 5×10^9 , 1×10^{10} , 5×10^{10} , 1×10^{11} cfu/ml whole cell suspensions were compared and the most optimal concentration was 1×10^8 cfu/ml.

The prevalence of *Campylobacter* spp

Of the 271 samples, the prevalence of *Campylobacter* spp in northern Thailand was found to be 46.5% *C. coli*, 35.8% *C. jejuni* and 8.9% other *Campylobacter* spp. Some samples (8.9%), which were confirmed as positive with microbiological method, did not give the 23S rRNA PCR amplified products.

The prevalence of *Campylobacter* at the farm showed that *C. jejuni* was the most prevalent species (42.5%) compared to *C. coli*, which was found at 39.1% and other *Campylobacter* spp (8.0%). In contrast, the prevalence at the slaughterhouse was found to be predominantly *C. coli* (72.4%), which was much higher than *C. jejuni* (17.2%) and other *Campylobacter* spp (3.4%). Similarly, at the market, the most prevalent species was *C. coli* (54.4%) while *C. jejuni* prevalence was 26.5% and other *Campylobacter* spp 13.2% (Table 3).

Moreover, *Campylobacter* spp isolated from farm workers were found to be predominantly *C. coli* (75%), while *C. jejuni* was found only for 25% isolates.

DISCUSSION

A total of 271 (65.3%) samples were recovered from the 415 frozen isolates. There could be several reasons that may affect recovery, such as the storage duration of the isolates in the freezer. We noted, for instance, that the recovery rates of the *Campylobacter* stocks of year 2002, 2001, and 2000 were 93.1%, 74.3%, and 40.6%, respectively. Identical methods and conditions were used

Table 2
The number of recovered *Campylobacter* isolates that were tested.

Source	Sample type	2000	2001	2002	Total
Farm	Cloacal swab	42/97	76/126	38/41	156/264
	Environment	-/-	-/1	14/14	14/15
	Worker	-/-	2/2	2/3	4/5
Slaughter house	Cloacal and carcass swab	16/46	52/53	-/-	68/99
Market	Meat	-/-	29/32	-/-	29/32
	Total	58/143 (40.6%)	159/214 (74.2%)	54/58 (93.1%)	271/415 (65.3%)

Table 3
The number of *Campylobacter* spp from each source.

Source	N ^a	Total	CC	CJ	Other	OR (CC:CJ)	p-value
Farm	425	156	68	74	14	0.85	0.57
Market	72	27	21	5	1	15.40	<0.01
Slaughter	352	64	37	18	9	3.50	<0.01
Grand total	849	247	126	97	24	1.93	<0.01

^a = The number of total collected samples

in the three years for freezing, thawing, and regrowing. Therefore, the recovery rates observed in our study suggest that long storage of *Campylobacter* in the freezer may decrease the survival of the organisms or weaken them in such away that very special methods of regrowing are needed in order to achieve a high percentage of recovery.

The results of this study showed that overall *C. coli* was the most prevalent (46.5%) species of *Campylobacter* from the different sources sampled. Comparing the prevalence of the different *Campylobacter* spp from each source sampled, however, showed that *C. jejuni* was the most prevalent species in samples taken from chickens at the farm and *C. coli* was the most prevalent in chicken samples taken at the slaughterhouse and at the market. Our results differ from those reported in the literature describing species of *Campylobacter* in chicken meat products. Saenz *et al* (2000) and Chattopadhyay *et al* (2001) reported that *C. jejuni* was the most prevalent in samples isolated from chicken and foods containing chicken products. In addition to the fact that these studies were done in different countries where the *Campylobacter* exposure to chickens or food with chicken products may be different from those in Thailand, the methods used to characterize *Campylobacter* isolates were also different. Saenz *et al* (2000) and Chattopadhyay *et al* (2001) used biochemical tests for identifying the *Campylobacter* species whereas we used a molecular technique (Multiplex PCR assay) to differentiate between *C. jejuni* and *C. coli*.

We isolated *Campylobacter* spp from 5 of the 129 (3.9%) chicken farmers and no *Campylobacter* was isolated from 100 neighboring crop farmers who served as controls, suggesting that the chick-

ens might have served as the source of *Campylobacter* to the chicken farmers. The majority of the isolates (75%) from the chicken farmers were *C. coli* and all the individuals from which *Campylobacter* was isolated were adults with no diarrhea or any other health conditions three months prior and on the days samples were collected. Our results differ from those reported in Thailand by Echeverria *et al* (1989) and Taylor *et al* (1991) where they found *C. jejuni* as the most prevalent *Campylobacter* spp in children with diarrhea. Our results also differ from those of Friedman *et al* (2000) who reported that up to 99% of *Campylobacter* enteritis cases were caused by *C. jejuni* in developed countries.

The results of our study indicate that both *C. jejuni* and *C. coli* are highly prevalent in chickens and along the chicken meat production system in northern Thailand. It is generally believed that pigs and pork products are the major sources of *C. coli* to humans. We can not explain the fact that *C. coli* was more prevalent in samples taken from the chickens at the slaughterhouse and fresh meat market. Cross contamination at the slaughterhouse and the fresh meat market is a possible explanation. In fact Rivoal *et al* (1999) and Newell *et al* (2001) discuss the possibility of environmental and cross contamination between batches of animals slaughtered at the slaughterhouse. Critical control points for exposure of chickens at the farm and contamination of chicken at slaughterhouses and the fresh markets need to be determined so that control measures can be implemented to protect the human food supply.

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