International Food Research Journal 16: 277-288 (2009)

Comparison of thermophilic *Campylobacter* spp. occurrence in two types of retail chicken samples

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Abstract: The aim of this study is to compare the occurrence of thermophilic *Campylobacter* spp. in chicken retail at wet markets and hypermarkets. *Campylobacter* contaminations in chicken samples from wet market (70.7%) were comparatively lower than chicken samples sold in hypermarket (91.4%). Of the 77 *Campylobacter* isolates, 59 (76.6%) were identified as *Campylobacter jejuni* and 18 (23.4%) isolates were identified as *C. coli.* All *Campylobacter* isolates are multi-resistant to the antimicrobial agents. Most of the isolates were resistant to tetracycline (92.2%) and erythromycin (98.7%). This study concluded that chicken samples from both wet market and hypermarket were contaminated with *Campylobacter,* most of which are antimicrobial-resistant strains.

Keywords: *Campylobacter*, hypermarket, wet market, chicken parts

Introduction

Campylobacter spp. infection in humans is significantly increasing and has been reported to exceed the number of cases of Salmonella infections (Phillipps, 1995). Food of animal origin is likely to be contaminated by *Campylobacter* spp. as they are carried in the intestinal tract of warm-blooded animals. Recently *Campylobacter* spp. had been reported to contaminate fresh produce like *Ulam* (Chai *et al.*, 2007) and ready to eat sushi (Tan *et al.*, 2008).

There are reports of high number of acute *Campylobacter* enteritis or campylobacteriosis in humans which had been implicated with the consumption of chicken meats and chicken products (CDC, 2005; Skirrow, 1998; Tauxe, 1992). Though fatalities caused by *Campylobacter* infections are rare, they may lead to serious autoimmune sequelae, such as Guillain Barré syndrome and neuropathy (Park *et al*., 1991). Black *et al*. (1988) reported that some *C. jejuni* strains are highly infectious with the infective dose to be as low as 800 cells. Thermophilic *Campylobacter* spp., particularly *C. jejuni* and *C. coli* have been recognized as the most important pathogenic strains within the genus due to their frequently isolation from infected persons (Skirrow, 1998).

In Malaysia, chicken parts are available in conventional wet markets and modern hypermarkets. Conventional wet markets set-up appear to be clean, simple and have less equipped facilities while modern hypermarkets set-up appear to be

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clean and have well-equipped facilities. Conventional wet markets are popular as they offer live chickens being slaughtered on-site but have short operating hours (~ 6 hours/day) in the morning. This ensures the chickens on sale are very fresh every day. Hypermarkets offer fresh chickens in chilled condition for longer storage time (2 – 3 days) and long operating hours (~ 12 hours/day). Wet markets' chickens are of the interest to those who want fresh chickens which had been just slaughtered as it had been thought to be healthier than chilled chickens. Hypermarkets' chickens however interest those who want convenience and don't have time to go wet market early in the morning but still want fresh chicken. Both wet markets and hypermarkets offer fresh chickens but the safety of the chickens in terms of *Campylobacter* spp. contamination is not known.

In this study, we aim to determine the prevalence of campylobacters in chicken parts retailed in wet markets and hypermarkets. We also want to compare the occurrence of thermophilic *Campylobacter* spp. between the wet markets and hypermarkets. To the best of our knowledge, this is the first study to compare the prevalence of campylobacters in retail chicken parts from two different retail outlets set-up.

Materials and Methods

Sample collection

A total of 185 samples of chicken samples were purchased from 4 wet markets and 3 hypermarkets. Ninety-three chilled chicken samples were purchased from hypermarkets. All chilled chicken parts were packed and stored/displayed at chiller for 2 to 3 days. Chilled samples were purchased on the first day of packaging (based on the packaging label) and transported on ice to the laboratory in separate containers. Ninety-two samples for fresh chicken parts were purchased from wet markets and transported to the laboratory in separate containers without ice. Table 1 showed the differences between the conditions in hypermarket and wet market and chicken samples from the retail outlets in general. All samples were protected from sunlight and processed within 2 hours after purchased. The temperature of samples was taken at the time of purchase.

Enrichment

For the recovery of campylobacters, 10 g of each chicken sample (including skin in case of breasts, keels, drumsticks, wings and bishops) were cut into small pieces (<0.25 cm^2) using sterile scalpel blade on sterile Petri dishes. Each sample was added into a stomacher bag containing 90 ml of Bolton Selective Enrichment Broth (BEBB; Merck, Darmstadt, Germany) supplemented with Bolton antibiotic supplements (Merck, Darmstadt, Germany) and 5% lysed horse blood. All Bolton enrichment broth was prepared fresh, cooled to room temperature in the dark and used within 12 h; Bolton antibiotic supplements and 5% lysed horse blood were added only prior to sample enrichment. The samples were mixed by hand for 30s and allowed to stand for 1 min. The homogenates were transferred to screw-capped sterile bottles leaving very little headspaces above the liquid. The bottles were then incubated in anaerobic jar under microaerophilic condition produced using Anaerocult C (Merck) at 42^oC for 48 h.

Campylobacter spp. isolation

From the enrichment bottles, 0.1 ml of the broth culture was plated on modified charcoal-cefoperazone-deoxycholate blood free selective agar (mCCDA; Merck) with antibiotic supplements in duplicates. The plates were incubated under microaerophilic condition generated by Anaerocult C (Merck)

at 42°C for 48 h. Presumptive identification of *Campylobacter* spp. colonies was based on the Gram-staining and colonial appearance as described by Stern *et al.* (2001). Colonies with consistent morphology with *Campylobacter* spp. were subcultured and were confirmed by biochemical test, such as motility, catalase and oxidase tests. *C. jejuni* and *C. coli* were confirmed by PCR using species-specific primers.

DNA extraction

DNA extraction from enrichment samples were carried as described by Chai *et al*. (2007) with modification. Portions of 1 ml of each positive sample were subjected to centrifugation at 15,000 x *g* for 5 min to pellet the microorganisms. The supernatant was discarded and the pellet was washed once with 500 µl sterile distilled water. The pellet was then resuspended in 500 µl of sterile TE buffer (pH8.0) by vigorous vortexing and boiled for 10 min to release the DNA from the microorganisms. The sample was later cooled at -20°C for 10 min. The cooled sample was again subjected to centrifugation at 15,000 x *g* for 5 min. 100 µl supernatant which contain DNA was transferred to a new sterile microcentrifuge tubes. These DNA samples were stored at -20°C until being determined for the presence of *Campylobacter* spp., *C. jejuni* and *C. coli* using PCR assay.

DNA extraction of *Campylobacter* cells from agar plates were the same as described above without pelleting the cells and washing steps.

PCR assay

All enriched samples were examined for the presence of *Campylobacter* spp., *C. jejuni* and *C. coli* by PCR assay. Three *Campylobacter* genes were selected for the identification of *Campylobacter* spp., *C. jejuni* and *C. coli* using the *16S* rRNA gene (Linton *et al*., 1996), the *hip* gene (Linton *et al*., 1997) and the *ceuE* gene (Gonzalez *et al*., 1997), respectively. Table 2 shows the sequences of the primers used for gene amplification. The oligonucleotide primers used in this study were synthesized by 1st BASE Laboratories, Malaysia. DNA from reference cultures, *C. jejuni* (ATCC 33560) and *C. coli* (ATCC 43478), were included as a positive control in every PCR assay.

PCR amplification was performed in 25 µl of a reaction mixture containing 5 µl of 5× PCR buffer; 0.2 mM of deoxynucleoside triphosphate mix; 0.4 µM of each primer; and 2 µl of DNA preparation. All items used in PCR assay were purchased from Promega, Madison, USA. PCR reaction mixtures were heated at 95°C for 2 min as an initial denaturation step followed by 30 cycles of denaturation at 95°C (30s), annealing (60s) and extension 72°C (40s). Annealing temperature for *Campylobacter* spp., *C. jejuni* and *C. coli* were 55°C, 59°C and 55°C respectively. All PCR assays were terminated with a 3 min extension at 72°C and were performed with Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

For visualization of PCR products, 5 µl of PCR products were run on 1.0% agarose gel at 90 V for 40 min. The gel was then stained with ethidium bromide and viewed under ultraviolet (UV) light. A DNA-molecular ladder (100-bp ladder) (Vivantis Technologies, Selangor, Malaysia) was included in each gel.

Antimicrobial susceptibility of Campylobacter spp.

A total of 77 isolates of *Campylobacter* spp. were isolated from chicken parts comprised of 59 *C. jejuni* and 18 *C. coli* isolates. All isolates were revived from glycerol stocks. Bolton enrichment broth supplemented with Bolton supplement (Merck KGaA, Darmstadt, Germany) and

Table 1. The description of the retail outlets and chicken samples that being studied

Characteristics	Wet market	Hypermarket
Type	Open-air	Enclosed
Appearance	Clean	Clean
Chickens' freshness	Fresh	Fresh
Chicken slaughtered on-site	Yes	No
Carcass chilling	No	Yes
Packaging	No packaging	Chicken parts on Styrofoam over-wrapped with polyethylene film
Display duration	~ 6 hours	2 – 3 days
Display condition	At ambient temperature on stainless steel tray	Chilled

Table 2. Primer sequences, $MgCl_2$ concentration, amount of *Taq* and PCR product size for the PCR amplification of *Campylobacter* spp., *C. jejuni* and *C. coli*

Targeting species	Targeting gene and primer's sequence	$MgCl_2$ conc. (mM)	Amount of *Taq* (U)	Product size (bp)	Reference
Campylobacter spp. (genus)	***16S* rRNA gene** C412F: 5'-GGA TGA CAC TTT TCG GAG C-3' C1288R: 5'-CAT TGT AGC ACG TCT GTC-3'	2.5	0.75	816	Linton *et al.*, 1996
C. jejuni	***hip* gene** HIP400F: 5'-GAA GAG GGT TTG GGT GGT G-3' HIP1134R: 5'-AGC TAG CTT CGC ATA ATA ACT TG-3'	2.5	0.75	735	Linton *et al.*, 1997
C. coli	***ceuE* gene** F: 5'-ATG AAA AAA TAT TTA GTT TTT GCA-3' R: 5'-ATT TTA TTA TTT GTA GCA GCG-3'	3.0	0.5	894	Gonzalez *et al.*, 1997

5% lysed horse blood were used to revive the cultures. They were incubated at 42°C for 48 hours under microaerophilic conditions produced using the Anaerocult C system (Merck KGaA, Darmstadt, Germany).

Antibiotic resistance patterns were determined using the disk diffusion method, according to the guidelines of The National Committee for Clinical Laboratory Standards (NNCCLS, 2003). All isolates were grown in Brain heart infusion (BHI; Oxoid, Hamphire, United Kingdom) for 24 hours and were swabbed using a sterile non-toxic swab on Mueller-Hinton (MH) agar plates (Merck KGaA, Darmstadt, Germany) to form a uniform lawn of bacterial growth. Antibiotic disks were placed on the surface of the agar using a disk dispenser. Thirteen antibiotics were selected for the tests. The 13 antibiotics were: ampicillin (10 µg), cephalothin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), erythromycin (15 µg), gentamycin

(10 µg), kanamycin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), penicillin G (10 iU), streptomycin (10 µg) and tetracycline (30 µg). Antibiotic cartridges with commercially prepared antibiotic disks were purchased from Oxoid (Hamphire, United Kingdom). All plates were incubated at 42°C for 48 hours under microaerophilic conditions produced using Anaerocult C system (Merck KGaA, Darmstadt, Germany). After incubation, the size of the inhibition zones was recorded and the levels of susceptibility (sensitive and resistant) were determined according to the NCCLS guidelines.

Statistical analysis

Prevalence of *Campylobacter* spp. from wet market and hypermarket chicken samples was subjected to Chi-square test using the Minitab Release 14.

Results

A total of 185 chicken samples, 77 (41.6%) samples were detected *Campylobacter*-positive using conventional plating method while molecular method (PCR) detected 154 (83.2%) samples were *Campylobacter*-positive. Detection of *Campylobacter* using molecular method (PCR) were significantly (P<0.05) higher than conventional plating method. Figure 1 shows a representative gel electrophoresis image of the PCR amplification of *16S* rRNA, *hip* gene and *ceuE* gene for *Campylobacter* spp., *C. jejuni* and *C. coli*.

Out of 93 hypermarkets and 92 wet markets chicken samples, the prevalence of *Campylobacter* occurrence was 91.4% and 70.7%, respectively. The mean temperature of the chicken samples retailed at hypermarkets and wet markets was 5.0°C and 29.6°C, respectively. The prevalence of *Campylobacter* contamination in chicken samples from the wet markets was significantly lower (P<0.05) than those from the hypermarkets.

The prevalence of *C. jejuni* and *C. coli* in chicken samples from hypermarket were 91.4% and 34.4%, respectively. The prevalence of *C. jejuni* and *C. coli* in chicken samples from wet market were 70.7% and 20.7%, respectively. The occurrence of *C. jejuni* and *C. coli* showed similar pattern with *C. jejuni* was significantly higher than *C. coli* (P<0.05) for chicken samples from both hypermarket and wet market. Table 3 summarized the prevalence of *C. jejuni* and *C. coli* in chicken parts from hypermarkets and wet markets.

In the current study, 77 *Campylobacter* isolates were prepared for susceptibility testing to thirteen antimicrobial agents (Table 4). The highest percentage of resistance was observed toward erythromycin (98.7%) and tetracycline (92.2%). Resistance towards quinolones, namely ciprofloxacin, enrofloxacin, norfloxacin, and nalidixic acid, were 81.8%, 70.1%, 75.3%, and 42.9% respectively. The lowest frequency of antibiotic resistance was observed toward gentamicin (35.1%).

Discussion

Contamination of *Campylobacter* spp. in poultry has been recognized worldwide (Son *et al*., 1996; Denis *et al*., 2001; Saleha, 2004; Havelaar *et al*., 2006; Sallam, 2007). Havellaar *et al*. (2006) reported guaranteed Campylobacter-free chicken meat at retail level is not realistic at this moment. In Malaysia, retail chickens are available in both hypermarket and wet market. Campylobacters occur in both hypermarket and wet market chicken samples at a high percentage with high occurrence of *C. jejuni* and low occurrence of *C. coli*. The findings of the present study

Table 3. Prevalence of *C. jejuni* and *C. coli* in chicken parts from hypermarket and wet market

Chicken parts	Hypermarket				Wet Market			
	C. jejuni		*C. coli*		*C. jejuni*		*C. coli*	
	No.	%	No.	%	No.	%	No.	%
Thigh & Drumsticks	10/11	90.9	4/11	36.4	10/11	90.9	3/11	27.3
Breasts	11/11	100.0	5/11	45.5	10/11	90.9	3/11	27.3
Wings	10/12	83.3	3/12	25.0	7/12	58.3	1/12	8.3
Keels	12/12	100.0	5/12	41.7	10/11	90.9	3/11	27.3
Livers	12/12	100.0	7/12	58.3	12/12	100.0	5/12	41.7
Gizzards	11/11	100.0	4/11	36.4	11/11	100.0	3/11	27.3
Feet	10/12	83.3	1/12	8.3	1/12	8.3	0/12	0.0
Bishops	9/12	75.0	3/12	25.0	4/12	33.3	1/12	8.3
Average	**85/93**	**91.4**	**32/93**	**34.4**	**65/92**	**70.7**	**19/92**	**20.7**

Table 4. Number and percentages of antimicrobial-resistant *Campylobacter* strains isolated from chicken samples

Antibiotic	*C. jejuni* (n = 59)	*C. coli* (n = 18)	Total (n = 77)
Ampicillin	57 (96.6%)	9 (50.0%)	66 (**85.7%**)
Cephalothin	35 (59.3%)	8 (44.4%)	43 (**55.8%**)
Ciprofloxacin	50 (84.7%)	13 (72.2%)	63 (**81.8%**)
Cloramphenicol	50 (84.7%)	15 (83.3%)	65 (**84.4%**)
Enrofloxacin	42 (71.2%)	12 (66.7%)	54 (**70.1%**)
Erythromycin	58 (98.3%)	18 (100.0%)	76 (**98.7%**)
Gentamicin	22 (37.3%)	5 (27.8%)	27 (**35.1%**)
Kanamycin	55 (93.2%)	12 (66.7%)	67 (**87.0%**)
Nalidixic Acid	23 (39.0%)	10 (55.6%)	33 (**42.9%**)
Norfloxacin	47 (79.7%)	11 (61.1%)	58 (**75.3%**)
Penicillin G	54 (91.5%)	14 (77.8%)	68 (**88.3%**)
Streptomycin	52 (88.1%)	11 (61.1%)	63 (**81.8%**)
Tetracycline	54 (91.5%)	17 (94.4%)	71 (**92.2%**)

are in close agreement with reports from different studies worldwide (Denis *et al*., 2001; Sallam, 2001; Whyte *et al*., 2004) in which *C. jejuni* is predominant while *C. coli* was less frequently encountered. Such high occurrence might be due to improper handling, contaminated water and cross-contamination in various stages of chickens' processing as well as packaging.

Campylobacter is known to be very sensitive to oxygen and require exact growth requirements in laboratory media. Detection of *Campylobacter* spp. on mCCDA agar plates in this study appears to be lower than detected by PCR assay. Detection of *Campylobacter* spp. from retail poultry varies greatly, from 0% to 71.2% in several reports (Willis and Murray, 1997; Cloak *et al*., 2001; Dominguez *et al*., 2002; Whyte *et*

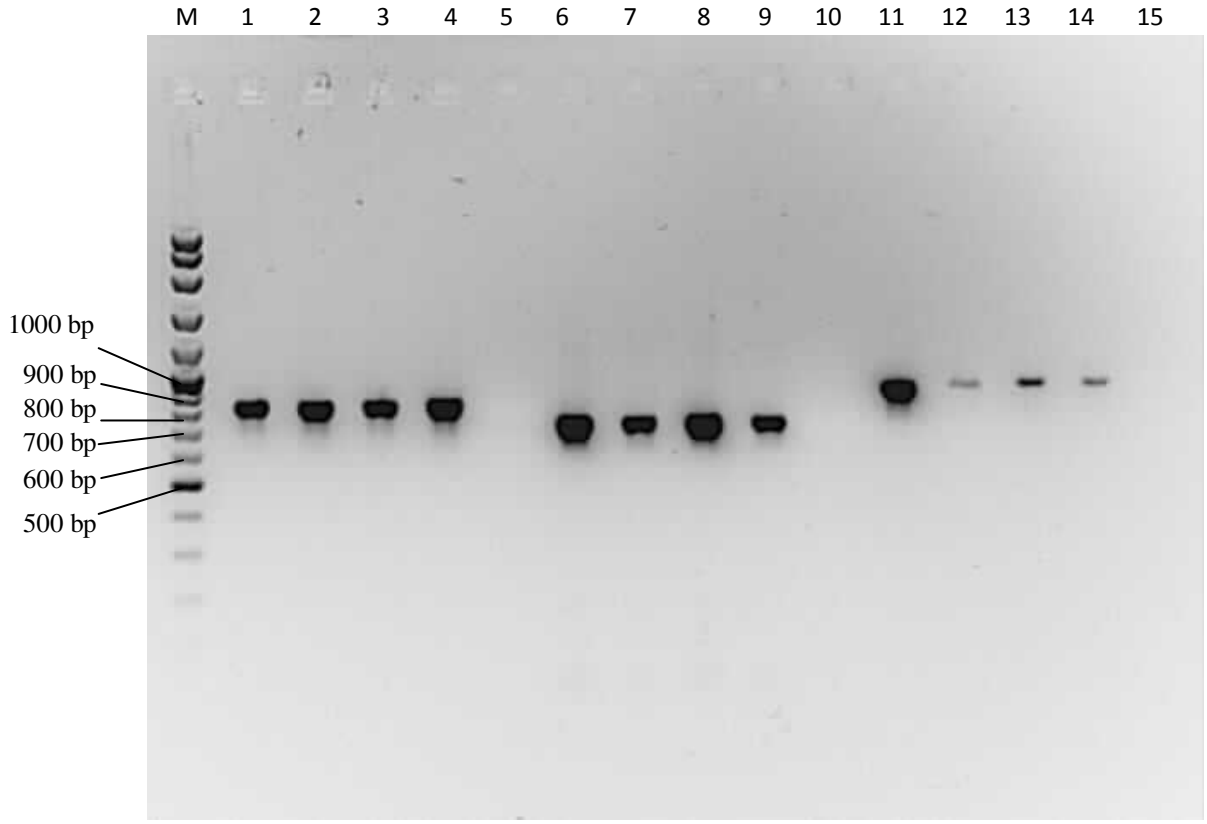

Figure 1. Representative amplification of the *16S* rRNA, *hip* genes and *ceuE* genes for identification of *Campylobacter* spp., *Campylobacter jejuni* and *Campylobacter coli* respectively. Lanes 1 to 4 show the PCR amplicons specific for *Campylobacter* spp. at 816 bp. Lanes 6 to 9 show the PCR amplicons specific for *C. jejuni* at 735 bp. Lanes 11 to 14 show the PCR amplicons specific for *C. coli* at 894 bp. Lane M shows the 100-bp DNA ladder, (1) *C. jejuni* reference strain (ATCC 33560), (2), (3) and (4) DNA from an enrichment broth, (6) *C. jejuni* reference strain (ATCC 33560), (7), (8), and (9) DNA from an enrichment broth, (11) *C. coli* reference strain (ATCC 43478), (12), (13), and (14) DNA from enrichment broth, (5), (10) and (15) negative control.

al., 2004; Saito *et al*., 2005). Currently there is no single method that is universally used in laboratories for detection and isolation of campylobacters from food and veterinary samples (Whyte *et al*., 2003), such variability is expected. Whyte *et al*. (2003) showed that isolation of *Campylobacter* spp. is media-dependent and Atabay and Corry (1997) reported filtration and enrichment method showed fewer recovery of *Campylobacter* spp. compare to direct streaking. Chai *et al*. (2007) also evidently showed higher sensitivity of PCR in detecting the presence of *Campylobacter* compare to conventional plating method.

Low recovery of *Campylobacter* found in this study might also be due to enrichment time for 48 h. Madden *et al*. (2000) reported extended enrichment (72 h) reduces recovery of Campylobacters. However, two other reports showed 24 h enrichment of chicken samples gave good recovery of *Campylobacter* spp. (Denis *et al*., 2001; Josefsen et al., 2004). Chai *et al*. (2007) reported similar low recovery of *Campylobacter* from vegetables after MPN-

enrichment for 48 h. Thus, enrichment incubation time may be reduced to 24 h to improve recovery of *Campylobacter* but study need to be done on its effect on PCR detection. Besides that, vancomycin which is part of Bolton antibiotic supplements used in this study was reported to have some inhibitory effect on campylobacters (Humphrey, 1990). All the above-mentioned stresses might explain low *Campylobacter* isolation from chicken samples in this study. The campylobacter cells which cannot be isolated were either in the viable but non-culturable (VBNC) state or dead.

Chickens from wet market showed lower prevalence of *Campylobacter* contamination compared to chickens from hypermarket. Wet market chicken samples with average temperature 29.6^{o}C are not favorable for *Campylobacter* spp. to grow or multiply as they do not grow outside 32–44^{o}C (Stanley *et al*., 1998). This added stress on *Campylobacter* which may contribute to the lower prevalence of *Campylobacter* from wet market chicken samples. In addition, *Campylobacter* spp. was seen to survive better in chilled condition (Reezal *et al*., 1998; Hänel and Atanassova, 2007) and this might explain the prevalence is higher compare to fresh samples with higher storage temperature in present study.

Resistance to antimicrobial agents in this study showed to be very high. This phenomenon had been seen worldwide (Chai *et al*., 2008; Saleha, 2002; Sallam, 2007; Taremi *et al*., 2006). Generally antimicrobial agents were massively used on intensively-reared chickens for therapy, prophylaxis and growth promotion (Pezzotti *et al*., 2003; Soonthornchaikul *et al*., 2006). Such approach may contribute to the transmission of antimicrobial-resistant *Campylobacter* to humans from chickens. *Campylobacter* resistances to antimicrobial agents were particularly concerned as quinolones and erythromycin had been widely use for human therapy. (Aasrestrup and Engberg, 2001; Engberg *et al*., 2004). Chai *et al*. (2008) and Tan *et al.* (2009) had reported high resistance of *Campylobacter* isolates in developing country such as Malaysia. This phenomenon might be due to abuse and misuse of antimicrobial agents in agricultural farming in Malaysia.

The present study showed there is high incidence of *Campylobacter* in chicken samples examined. This indicates that chickens might be commonly contaminated with campylobacters; most of which were antimicrobial-resistant. Thus, it might pose a serious health risk to consumers who consumed undercooked or post-cooking contaminated chickens as antibiotics, namely erythromycin or tetracycline, are normally being prescribed in serious campylobacteriosis in human cases such as bloody diarrhea and blood infection in immuno-compromised patients. With the increase of *Campylobacter* resistance towards antibiotics, the antibiotics treatment in such cases will be compromised.

Acknowledgements

This study was supported by Science Fund (project no. 05-01-04-SF0379) from the Ministry of Science, Technology and Innovation, Malaysia and in-part by Grant-in-Aid for Scientific Research (KAKENHI 191010) from Japan Society for the Promotion of Sciences. We wish to thank Faculty of Veterinary, UPM for generously supplying the fresh horse blood.

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