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

Tang, J.Y.H., 1* Mohamad Ghazali, F., 2 Saleha, A.A., 3 Nishibuchi, M. and 1 Son, R.

1 Centre of Excellence for Food Safety Research, Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
2 Department of Pathology-Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
3 Center for Southeast Asian Studies, Kyoto University, Yoshida, Sakyo-ku, Kyoto, Japan

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 (Phillips, 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
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 cm²) 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°C 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)
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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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Wet market</th>
<th>Hypermarket</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Open-air</td>
<td>Enclosed</td>
</tr>
<tr>
<td>Appearance</td>
<td>Clean</td>
<td>Clean</td>
</tr>
<tr>
<td>Chickens’ freshness</td>
<td>Fresh</td>
<td>Fresh</td>
</tr>
<tr>
<td>Chicken slaughtered on-site</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Carcass chilling</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Packaging</td>
<td>No packaging</td>
<td>Chicken parts on styrofoam over-wrapped with polyethylene film</td>
</tr>
<tr>
<td>Display duration</td>
<td>~ 6 hours</td>
<td>2 – 3 days</td>
</tr>
<tr>
<td>Display condition</td>
<td>At ambient temperature on stainless steel tray</td>
<td>Chilled</td>
</tr>
</tbody>
</table>

Table 2. Primer sequences, MgCl₂ concentration, amount of Taq and PCR product size for the PCR amplification of *Campylobacter* spp., *C. jejuni* and *C. coli*

<table>
<thead>
<tr>
<th>Targeting species</th>
<th>Targeting gene and primer’s sequence</th>
<th>MgCl₂ conc. (mM)</th>
<th>Amount of Taq (U)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter</em> spp. (genus)</td>
<td>16S rRNA gene C412F: 5’-GGA TGA TAC TTT TCG GAG C-3’ C1288R: 5’-CAT TGT AGC ACG TCT GTC-3’</td>
<td>2.5</td>
<td>0.75</td>
<td>816</td>
<td>Linton et al., 1996</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>hip gene HIP400F: 5’-GAA GAG GGT TTG GGT GGT G-3’ HIP1134R: 5’-AGC TAG CTT CGC ATA ATA ACT TG-3’</td>
<td>2.5</td>
<td>0.75</td>
<td>735</td>
<td>Linton et al., 1997</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>ceuE gene F: 5’-ATG AAA AAA TAT TTA GTT TTT GCA-3’ R: 5’-ATT TTA TTA TTT GTA GCA GCG-3’</td>
<td>3.0</td>
<td>0.5</td>
<td>894</td>
<td>Gonzalez et al., 1997</td>
</tr>
</tbody>
</table>

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). Havelaar 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

<table>
<thead>
<tr>
<th>Chicken parts</th>
<th>Hypermarket</th>
<th></th>
<th></th>
<th>Wet Market</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. jejuni</em></td>
<td><em>C. coli</em></td>
<td></td>
<td><em>C. jejuni</em></td>
<td><em>C. coli</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Thigh &amp; Drumsticks</td>
<td>10/11</td>
<td>90.9</td>
<td>4/11</td>
<td>36.4</td>
<td>10/11</td>
<td>90.9</td>
</tr>
<tr>
<td>Breasts</td>
<td>11/11</td>
<td>100.0</td>
<td>5/11</td>
<td>45.5</td>
<td>10/11</td>
<td>90.9</td>
</tr>
<tr>
<td>Wings</td>
<td>10/12</td>
<td>83.3</td>
<td>3/12</td>
<td>25.0</td>
<td>7/12</td>
<td>58.3</td>
</tr>
<tr>
<td>Keels</td>
<td>12/12</td>
<td>100.0</td>
<td>5/12</td>
<td>41.7</td>
<td>10/11</td>
<td>90.9</td>
</tr>
<tr>
<td>Livers</td>
<td>12/12</td>
<td>100.0</td>
<td>7/12</td>
<td>58.3</td>
<td>12/12</td>
<td>100.0</td>
</tr>
<tr>
<td>Gizzards</td>
<td>11/11</td>
<td>100.0</td>
<td>4/11</td>
<td>36.4</td>
<td>11/11</td>
<td>100.0</td>
</tr>
<tr>
<td>Feet</td>
<td>10/12</td>
<td>83.3</td>
<td>1/12</td>
<td>8.3</td>
<td>1/12</td>
<td>8.3</td>
</tr>
<tr>
<td>Bishops</td>
<td>9/12</td>
<td>75.0</td>
<td>3/12</td>
<td>25.0</td>
<td>4/12</td>
<td>33.3</td>
</tr>
<tr>
<td>Average</td>
<td>85/93</td>
<td>91.4</td>
<td>32/93</td>
<td>34.4</td>
<td>65/92</td>
<td>70.7</td>
</tr>
</tbody>
</table>

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
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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°C are not favorable for Campylobacter spp. to grow or multiply as they do not grow outside 32–44°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.

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