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REGULAR ARTICLES

Occurrence of *Campylobacter* species from broiler chickens and chicken meat in Malaysia

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Abstract

Campylobacter is reported as a major cause of foodborne illness worldwide. Consumption of contaminated chicken meat is considered a significant risk factor of *Campylobacter* infection in humans. This study investigated the occurrence of non-*Campylobacter jejuni-Campylobacter coli*, in broiler chickens ($n = 210$) and chicken meat ($n = 109$). The samples were collected from seven broiler chicken farms ($n = 210$ cloacal swabs), 11 markets ($n = 84$ chicken meat), and 5 supermarkets ($n = 25$ chicken meat) located in different districts of Selangor State. *Campylobacter* were isolated from cloacal swabs using the Cape Town Protocol and from meat samples using the method of Duffy et al. (2007) with some modifications for *Campylobacter* isolations which were reported effective in the isolation of non-*C. jejuni-C. coli Campylobacter* species. The isolates were identified by Gram staining for cellular morphology, wet mount for motility and biochemical tests. Confirmation of presumed *Campylobacter* isolates was carried out using multiplex PCR (mPCR). One hundred seven (107/210) or 50.9% and twenty-nine (29/109) or 26.6% of chickens and chicken meat samples respectively were positive for *Campylobacter* species. Among the *Campylobacter* isolates from chickens, *C. jejuni* was the most predominantly isolated species (69.5%), followed by *C. coli* (16.2%). *Campylobacter fetus* and *C. upsaliensis* were the non-*C. jejuni-C. coli Campylobacter* species isolated in this study, at 9.3% and 2.5% respectively. Overall, the findings indicated broiler chickens were colonized not only by the common *Campylobacter* species but also by other *Campylobacter* species. We found the Cape Town Protocol useful to detect the occurrence of non-*C. jejuni-C. coli* isolates in chickens.

Keywords *Campylobacter* · Cape Town Protocol · Non-C. *jejuni-C. coli* · Malaysia

Introduction

Campylobacter species are increasingly being recognized as major causes of bacterial gastroenteritis in humans. The pathogens have also been associated with several clinical conditions in humans such as hemolytic-uremic syndrome, bacteremia, reactive arthritis, and pancreatitis (Linton et al. 1996). Currently, at the time of this writing, there are 34 species and 14 subspecies of genus *Campylobacter* that have been identified as listed in http://www.bacterio.net/ (Van et al. 2016; Kaakoush et al. 2015; Man 2011). Among these species, *Campylobacter jejuni* and *C. coli* are the most commonly isolated species from humans. However, an increasing number of *Campylobacter* species other than *C. coli* and *C. jejuni* have been reported and recognized as human and animal pathogens; they are known as non-*C. jejuni-C. coli Campylobacter* species (Lastovica and Allos 2008) which according to Kaakoush et al. (2015) is a term used to describe their under appreciated roles in human and animal diseases. These non-*C. jejuni-C. coli Campylobacter* species have the potential to contribute in the cause of gastroenteritis by unknown pathogens and they included *C. ureolyticus*, *C. upsaliensis*, *C. lari*, *C. fetus*, and *C. concisus.* Lastovica and le Roux (2000) isolated *C. concisus* (5%) and *C. upsaliensis* (4.9%) from 19,535 diarrheic stool samples obtained from

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children over 10-year period. Vandenberg et al. (2006) also reported the occurrence of *Campylobacter* species other than *C. coli* and *C. jejuni* from 269 out of 42,287 patients.

The role of *Campylobacter* species other than *C. jejuni* and *C. coli* or non-C. *jejuni-C. coli* in clinical diseases in humans has not been fully understood; this is due to isolation of fewer strains and differences in inter-laboratory detection methods (Lastovica 2006). Routinely used laboratory methods allow only the growth of *C. coli and C. jejuni* and seldom other *Campylobacter* species. The primary incubation temperature employed by most diagnostic laboratories is 42 °C. This temperature is suitable for *C. jejuni* and *C. coli* but not for other species, such as *C. fetus* or *C. hyointestinalis*, that grow at 37 °C (Lastovica 2006). Additionally, the use of antibiotics in the formulation of the selective media may inhibit the growth of some *Campylobacter* species (Corry et al. 1995).

The Cape Town Protocol is one of the most common and routinely used methods for the detection of almost all known *Campylobacter* species (Diergaardt et al. 2003; Lastovica 2006). The protocol employs the use of filtration through a membrane filter onto antibiotic-free blood agar plate with subsequent incubation at 37 °C in hydrogen-enriched microaerobic atmosphere. Recovery rate of up to 21.8% of *Campylobacter* from human stool sample has been reported (Lastovica 2006). To date, there are limited published reports on the use of this method to isolate *Campylobacter* from animal feces.

Campylobacter is commonly found in the gastrointestinal tract of poultry and is considered a commensal microorganism (Newell and Fearnley 2003). Some researchers regarded poultry as a natural reservoir of *Campylobacter* and its body temperature is suitable for the growth of *Campylobacter*. Poultry meat is well known major sources of human foodborne illnesses attributed to *Campylobacter* (Corry and Atabay 2001). Hence, this study was carried out to determine the occurrence of non-*C. jejuni-C. coli Campylobacter* species in broiler chickens and chicken meat retailed in markets in Malaysia.

Materials and methods

Sample collection

A total of 210 chickens of marketing age were sampled from seven broiler chicken farms and 109 chicken meat samples purchased from 11 markets ($n = 84$) and 5 supermarkets ($n = 25$) located in different districts of Selangor State, Malaysia. In each farm, 30 chickens were randomly sampled by cloacal swabbing. The cloacal swab was placed individually in a bottle containing sterile normal saline (2 mL), kept in a cool box packed with ice. In two of the farms, chickens were reared under a closed-house system and the other five farms had chickens raised in an open-sided housing systems. At the markets and supermarkets, chicken meat samples consisted of thighs, wings, and breasts; in each market, 4 to 10 chicken meat samples were purchased depending on the number of chicken meat stalls available in the markets while 5 samples were purchased per supermarket. The chicken meat at the market was displayed openly on countertops in the warm market environment. Those from the supermarkets were kept chilled in refrigerated cabinets or placed on countertops and covered with ice. Upon purchase, each meat sample was placed in a sterile plastic bag, kept in a cool box packed with ice. Samples were transported to the Veterinary Public Health Laboratory, Universiti Putra Malaysia. The cultures of the cloacal swabs and chicken meat samples were done within 3 h after collection.

Ethical compliance

This study was performed per the guidelines for the care and use of animals by Institutional Animal Care and Use Committee of UPM and Animal Welfare Act. In this study, the chickens in the farms were handled with care by the farm owners or workers and who also held the chickens while cloacal swabs were taken.

Bacterial isolation

The isolation of emerging *Campylobacter* from chickens (cloacal swabs) was carried out using the Cape Town Protocol developed by le Roux and Lstovica (1998) and Lastovica (2006). To each bottle which contained a cloacal swab, sterile saline was added and vortexed gently. A membrane filter (Schleicher & Schuell ME 26) of pore size 0.6 μm was aseptically placed onto the surface of each Tryptic blood agar (TBA, Oxoid) plate and three aliquots of 100 μL of each suspension were dropped onto the membrane filter which was then left to filter passively for 45 min. The membrane filters were removed and plates were covered. Although the protocol called for incubation of inoculated agar plates at 37 °C in H_2-enriched microaerobic atmosphere, however in this study, two different incubation temperatures were used. Thus, two sets of TBA plates were prepared for each sample with the agar containing 10% unlysed horse blood. A set of plates were incubated in H_2-enriched microaerobic condition generated by anaerobic gas pack without catalyst (Oxoid BR 0038B) at 37 °C for 6 days and the other set under microaerobic condition generated by CampyGen gas pack (Oxoid CN0025A). All plates were examined every 48 h and presumptive isolates were collected for identification and confirmation.

For the isolation of non-*C. jejuni-C. coli Campylobacter* from chicken meat, the procedure of Duffy et al. (2007) was used with some modifications. Briefly, 25 g of each meat sample was placed into a sterile plastic bag containing 225 mL Bolton broth (Oxoid) (*Campylobacter* Enrichment

Broth, CEB, of Lab M, UK was unavailable in the country) with 5% lysed horse blood and was then homogenized for 1.5 min using a stomacher. The homogenates were incubated at 37 °C microaerobically generated by CampyGen gas pack (Oxoid CN0025A) for 24 h. Subsequently, the homogenates were filtered and plated on TBA plates as above for cloacal swab samples.

Phenotypic identification of *Campylobacter* isolates

Three to five *Campylobacter*-like colonies (small, round, creamy-gray, or whitish colonies) from each TBA plate were selected and sub-cultured onto a Colombia Blood Agar (CBA, Oxoid) plate and incubated in H_2-enriched microaerobic environment at 37 °C for 48 h. Presumptive *Campylobacter* isolates were then selected and identified by the typical corkscrew motility upon the examination of wet mounts of suspected colonies, typical cellular appearance (curved rod/S-shaped, Gram negative) on Gram stain, and biochemical tests which included hippurate hydrolysis, nitrate reductase, indoxyl-acetate hydrolysis, urease, oxidase, catalase test, and H_2 production on Triple Sugar Iron agar. Phenotypically identified *Campylobacter* isolates were then preserved in FBP medium (Gorman and Adley 2004) at − 80 °C until confirmation and speciation using multiplex PCR (mPCR) assay.

Genomic DNA extraction and PCR detection of *Campylobacter* isolates

Bacterial DNA was extracted from fresh cultures of *Campylobacter* incubated at 42 °C for 48 h in a microaerophilic environment. A suspension of a few pure colonies of *Campylobacter* isolates were prepared in a 1.5-mL sterile micro centrifuge tube containing 100 μL sterile distilled water and genomic DNA extraction was performed using the Wizard Genomic DNA purification kit (Promega) according to manufacturer's instruction. The DNA quality was visually evaluated after gel electrophoresis [1% (*w*/*v*) Agarose LE (Promega) gel in 1× Tris borate EDTA (TBE) (Bio basic Canada Inc.) stained with ethidium bromide] of 5 μL for each isolate at 100 V for 60 min and subsequently viewed under UV light. DNA preparations were stored at − 20 °C until use in the mPCR assay. Genotypic identification and confirmation of *Campylobacter* isolates were carried out using the mPCR assay as described by Yamazaki-Matsune et al. (2007) and Man et al. (2009). These protocols were used for identification of genus *Campylobacter*, species *C. jejuni*, *C. coli*, and non-*C. jejuni*-*C. coli Campylobacter* species namely *C. lari*, *C. helveticus*, *C. fetus*, *C. hyointestinalis* subsp. *hyointestinalis*, *C. upsaliensis*, and *C. concisus* (Table 1). The mPCR cycling condition was optimized using DNA extracts from known strains of *C. jejuni* (ATCC 29428), *C. coli* (ATCC 33559), *C. lari* (CCUG 23947T), *C. fetus* (CCUG 6823AT), and *C. upsaliensis* (CCUG 14913T). PCR amplifications were performed in a 50-μL reaction volume containing 25 μL [2× Master Mix (QIAGEN)], 5 μL [10× primer mix (2 μM of each primer)], 16 μL of RNase-free water (QIAGEN), and 4 μL of DNA template. The reaction mixtures were amplified in a DNA thermal cycler (Eppendorf) with the following cycling condition: initial denaturation for 15 min at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 1.5 min, and extension at 72 °C for 1 min, ending with a final extension step at 72 °C for 7 min.

Table 1 Primers and oligonucleotide sequence for the identification of *Campylobacter* species

Species	Size (bp)	Primer	Sequence (5′ to 3′)	References
Genus *Campylobacter*	816	CH412F C1228R	5′-GGATGACACTTTTCGGAGC-3′ 5′-CATTGTAGCACGTGTGTC-3′	Man (2011)
Campylobacter fetus	359	MG3F	5′-GGTAGCCGCAGCTGCTAAGAT-3′	Hum et al. (1997)
		CF359R	5′-AGCCAGTAACGCATATTATAGTAG-3′	Wang et al. (2002)
Campylobacter lari	251	CLF CLR	5′-TAGAGAGATAGCAAAAGAGA-3′ 5′-TACACATAATAATCCCACCC-3′	Lastovica and Allos (2008)
Campylobacter upsaliensis	86	CU61F CU146R	5′-CGATGATGTGCAAATTGAAGC-3′ 5′-TTCTAGCCCCTTGCTTGATG-3′	Lastovica and Allos (2008)
Campylobacter helviticus	1225–1375	CHCU146F CH1371R	5′-GGGACAACACTTAGAAATGAG-3′ 5′-CCGTGACATGGCTGATTCAC-3′	Linton et al. (1996)
Campylobacter hyointestinalis	1287	CFCH57F CH1344R	5′-GCAAGTCGAACGGAGTATTA-3′ 5′-GCGATTCCGGCTTCATGCTC-3′	Linton et al. (1996)
Campylobacter jejuni	735	HIP400F HIP1134R	5′-GAA GAG GGT TTG GGT GGT G-3′ 5′-AGC TAG CTT CGC ATA ATA ACTTG-3′	Linton et al. (1997)
Campylobacter coli	894	F R	5′-ATG AAA AAA TAT TTA GTT TTT GCA-3′ 5′-ATT TTA TTA TTT GTA GCA GCG-3′	Gonzalez et al. (1997)
Campylobacter concisus	560	Concisus F	5′-CTT GTG AAA TCC TAT GGC TTA-3′	Man et al. (2009)
		Concisus R	5′-CTC ATT AGA GTG CTC AGC C-3′	

Presumptive *Campylobacter* isolates were held at 4 °C prior to analysis. Amplified mPCR products were electrophoresed through 1.5% (*w*/*v*) agarose in 1× TBE buffer, and visualized by UV transillumination after staining with ethidium bromide.

Data analysis

The data analysis on the proportions of positive samples detected in the two types of farms and upon using two incubation atmospheric conditions was done. The analysis was carried out using Chi-Square test. A P value ≤ 0.05 is considered statistically significant.

Results

The overall occurrence of *Campylobacter* species in chickens in the farms was 107/210 (50.9%) (Table 2). More than one *Campylobacter* species were isolated from 11 chickens resulting in 118 *Campylobacter* isolates. The occurrence of *Campylobacter* in the seven broiler chicken farms ranged from 0 to 86.7% with a mean of 50.9%. The proportion of *Campylobacter*-positive birds in four farms was more than 50.0%, one farm showed 46.7% positive, one farm was found negative (0%) for *Campylobacter*, while another had very low occurrence (6.7%); these last two farms practiced closed-housing systems. Eighty-two (82) of the *Campylobacter*-positive isolates (69.5%) in the five farms were identified as *C. jejuni*, while 19 isolates (16.2%) were *C. coli* that were found in four farms. *C. fetus* and *C. upsaliensis* were the only non-*C. jejuni*-*C. coli Campylobacter* species isolated at 11 (9.3%) and three isolates (2.5%) respectively. The chickens that were found to carry more than one *Campylobacter* species were as follows: seven chickens were colonized by *C. jejuni* and *C. coli*, two chickens by *C. jejuni* and *C. fetus*, and two chickens by *C. coli* and *C. fetus*. Three isolates (2.5%) could not be identified at species level. Table 3 shows the isolation of *Campylobacter* when incubated under H_2-enriched microaerobic and microaerobic conditions at 37 °C. A higher rate of *Campylobacter* isolations was obtained when plates were incubated under microaerobic (57%) compared with H_2-enriched microaerobic (42.8%) conditions. The occurrence of *Campylobacter*-positive chicken meat in markets and supermarkets and the species identified were shown in Table 4. Overall, the occurrence of *Campylobacter* in meat was 29/109 (26.6%), of which 12/84 (14.3%) retailed in markets were *Campylobacter*-positive and 17/25 (68%) of those retailed in the supermarkets were *Campylobacter*-positive. *C. jejuni* was the main species isolated 20/29(68.9%), followed by *C. coli* 5/29(17.2%). *C. fetus was* the only non-*C. jejuni*-*C.coli Campylobacter* species isolated 4/29(13.7%) (Table 4).

Discussion

Campylobacter species have gained global notoriety as one of the most important causes of foodborne gastroenteritis in humans and this is further complicated by the rise in the number of multiple drug–resistant *Campylobacter* species and their presence in a number of animal reservoirs. In this study, the overall prevalence of *Campylobacter* in broiler chickens in the seven farms was 50.9%. The prevalence ranged from 46.7 to 86.7% in chickens raised in five open-sided house systems, while 0% and 6.7% in chickens raised in two closed-house systems. The result was comparable with the previous study by Huat et al. (2010) which found 85% of chickens raised in open-sided house systems were *Campylobacter*-positive while none of the chickens sampled from the closed-house systems was found to be positive for *Campylobacter*. The result of this study was higher than the 32/210 (15.2%)

Table 2 Occurrence of *Campylobacter* species in broiler chickens in seven farms

Number of samples per farm*	Number of *Campylobacter*-positive chickens (%)	*C. jejuni* (No.)	*C. coli* (No.)	*C. fetus* (No.)	*C. upsaliensis* (No.)	Other *Campylobacter* species (No.)
Farm 1 (30)	22 (73.3)	18	0	2	1	1
Farm 2 (30) (CHS)	2 (6.7)	0	0	1	0	1
Farm 3 (30)	20 (66.7)**	16	4	2	1	0
Farm 4 (30)	14 (46.7)**	12	1	1	1	0
Farm 5 (30)	26 (86.7)**	16	13	3	0	0
Farm 6 (30)	23 (76.7)**	20	1	2	0	1
Farm 7 (30)(CHS)	0 (0)	0	0	0	0	0
Total 210*	107 (50.9%)	82	19	11	3	3

*No. of chicken were 30 per farm

**A number of chickens were colonized by more than one *Campylobacter* species

CHS, closed-housing system

Table 3 *Campylobacter* species isolated from broiler chickens using two different atmospheric conditions during incubation

Campylobacter species	Atmospheric conditions	
	H_2-enriched microaerobic	Microaerobic
C. jejuni	41	74
C. coli	17	5
C. fetus	4	7
C. upsaliensis	3	0
Other *Campylobacter* species	1	2
Total	66	88

prevalence of *Campylobacter* species from retailed chicken meats and swabs of weighing scales and cutting boards reported by Ibrahim et al. (2018).

Campylobacter jejuni (68.9%) was the predominant *Campylobacter* species isolated from chicken meat, followed by *C. coli* (17.2%). This result was similar to most studies worldwide that reported *C. jejuni* as the most prevalent species isolated from chickens followed by *C. coli* (Newell and Fearnley 2003; Wainø et al. 2003; Salihu et al. 2008; Yousif et al. 2019). It is interesting that this study also isolated two non-*C. jejuni*-*C. coli Campylobacter* species, namely *C. fetus* (9.3%) and *C. upsaliensis* (2.5%). The species of three isolates could not be identified. It is possible that they could belong to other species apart from the ones expected and thus not included in the mPCR assay. Rossi et al. (2009) and Van et al. (2016) had reported the isolation of *C. avium* and *C. hepaticus* respectively from chickens.

The study found 26.6% of chicken meat positive for *Campylobacter*. The occurrence in meat from open, wet markets ranged from 0 to 30% while chilled meat in the supermarkets showed 60–80% positive. The *Campylobacter* isolated from chicken meat were identified mainly as C. *jejuni*. An earlier study had also reported the higher occurrence of *Campylobacter* in chicken meat retailed in hypermarkets (91.4%), while those retailed in wet markets were lower (70.7%) (Kottawatta et al. 2017).

Campylobacter fetus has been isolated from animals such as cattle, horse, and sheep (Man 2011). However, not many studies had reported the presence of *C. fetus* in poultry. *C. fetus* was isolated from turkey (Logue et al. 2003), duck (Ridsdale et al. 1998), and chicken (Kuana et al. 2008). According to Kempf et al. (2006), *C. fetus* of human origin is not able to colonize chickens, and that broiler chickens do not play a dominant role in human campylobacteriosis caused by *C. fetus*. *C. upsaliensis*

Table 4 *Campylobacter*-positive chicken meat in markets and supermarkets and the species identified

	No. of *Campylobacter*-positive meat (%)	*C. jejuni*	*C. coli*	*C. fetus*
Wet markets ($n = 84$)				
Market 1 (9)*	0	0	0	0
Market 2 (10)	0	0	0	0
Market 3 (10)	2 (20)	0	0	2
Market 4 (10)	3 (30)	2	1	0
Market 5 (5)	0	0	0	0
Market 6 (4)	0	0	0	0
Market 7 (10)	1 (10)	1	0	0
Market 8 (6)	1(16.6)	1	0	0
Market 9 (10)	4 (40)	2	1	1
Market 10 (5)	0	0	0	0
Market 11 (5)	1 (20)	1	0	0
	Subtotal = 12 (14.3)	7	2	3
Supermarkets ($n = 25$)				
Supermarket 1**	3 (60)	2	1	0
Supermarket 2	4 (80)	3	1	0
Supermarket 3	3(60)	2	1	0
Supermarket 4	4(80)	3	0	1
Supermarket 5	3(60)	3	0	0
	Subtotal = 17 (68)	13#	3	1
TOTAL, $n = 109$	29 (26.6)	20 (68.9)	5 (17.2)	4 (13.8)

*No. of samples/wet market

**5 samples/supermarket

#4 samples were contaminated by more than one *Campylobacter* species

is a catalase negative/weak *Campylobacter* which is commonly found in dogs and cats (Bourke et al. 1998; Goni et al. 2017). Even though the study on the presence of *C. upsaliensis* in other animals is still limited, the occurrence of this organism in broiler chickens has been reported in Nigeria; 31 (3.7%) of 828 *Campylobacter*-positive samples isolated from chicken were identified as *C. upsaliensis* (Salihu et al. 2008) and in Brazil and 4.3% *C. upsaliensis* was isolated from broiler chickens (Kuana et al. 2008).

C. fetus and *C. upsaliensis* are discovered as human pathogens and are included as non-*C. jejuni*-*C. coli Campylobacter* species (Vandenberg et al. 2006). *C. fetus* and *C. upsaliensis* have been isolated from patients with diarrhea (Man et al. 2009; Bullman et al. 2012) and *C. upsaliensis* has been regarded as the most important *Campylobacter* species after *C. jejuni* and *C. coli* causing human gastroenteritis (Bourke et al. 1998). These *Campylobacter* species have been reported to cause extra-gastrointestinal infections in humans. *C. fetus* has been reported to cause bacteremia and septicemia in human (Howe et al. 1995; Pacanowski et al. 2008). Gaudreau and Lamothe (1992) isolated *C. upsaliensis* from breast abscess.

This study reported the isolation of some non-*C. jejuni*-*C. coli Campylobacter* species which may cause the occurrence of diseases in humans. Molecular methods have been reported to have discriminatory power in detecting the presence of fastidious *Campylobacter* which are unable to grow in conventional plating methods (Bullman et al. 2012). It is suggested for future research that molecular methods such as PCR may be applied along with conventional plating methods.

Conclusion

From this study, it was shown that *Campylobacter* species other than *C. jejuni* and *C. coli* could be found in broiler chickens. These species could not be detected before because the methods commonly used in diagnostic laboratories were inadequate, such as the use of antibiotic-containing selective media, inappropriate isolation temperature, isolation atmospheric condition, and length of incubation period for isolation. These non-*C. jejuni*-*C. coli Campylobacter* species are also a concern to public health because they can cause disease to human such as diarrhea and septicemia.

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Compliance with ethical standards This study was performed per the guidelines for the care and use of animals by Institutional Animal Care and Use Committee of UPM and Animal Welfare Act.

Conflict of interest The authors declare that they have no conflict of interest.

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