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Prevalence of multidrug resistance *Campylobacter jejuni* and *Campylobacter coli* in chickens slaughtered in selected markets, Malaysia

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Abstract. The objectives of this study were to determine the occurrence of *Campylobacter* spp. in live chickens sold at wet markets in Selangor, Malaysia and the multidrug resistance (MDR) profiles of the isolates. Cloacal swabs were taken from the chickens before slaughter and their caecal mucosae were swabbed after slaughter. Of the 90 chickens examined, 68 (75.6%) were positive for *Campylobacter*. *Campylobacter* were recovered from caecal swabs (53/90) and cloacal swabs (34/90) and *Campylobacter coli* (46 isolates) were identified slightly more than *Campylobacter jejuni* (41 isolates), but these differences were not significant ($p<0.05$). The most frequently observed resistance was to cephalothin (95.5%), followed by tetracycline (80.8%), erythromycin (51.4%), enrofloxacin (42.4%) and gentamicin (24.4%). Multidrug resistance (resistant to four or more antibiotics) was detected in 35.3% isolates. *Campylobacter jejuni* showed nine resistance profiles and the most common was to gentamicin-eryhtromycin-enrofloxacin-cephalothin-tetracycline (32.4%) combination while *C. coli* showed six profiles, with cephalothin-tetracycline (32.2%) combination being most common.

INTRODUCTION

Campylobacter spp. are major food borne bacteria causing enteric disease in humans worldwide (Andersen *et al.*, 2006; Han *et al.*, 2007). Numerous reports in many parts of the world have shown the organisms to be most prevalent in chickens (Corry & Atabay, 2001; Humphrey *et al.*, 2007) with caeca, colon and cloaca of the birds as the main sites of colonization (Sahin *et al.*, 2002; Humphrey *et al.*, 2007). Poultry meat is regarded as the primary source of *Campylobacter* in human infection. *Campylobacter jejuni* and *Campylobacter coli* are the two species most commonly associated with enteric disease in humans (Han *et al.*, 2007; Humphrey *et al.*, 2007). In developed countries, young adults are mainly affected and in developing countries, the disease is most prevalent among children (Coker *et al.*, 2002). *Campylobacter* infections are most often self-limiting but may lead to serious consequences, such as the development of Guillian-Barre syndrome, reactive arthritis and irritable bowel syndrome (Han *et al.*, 2007; Humphrey *et al.*, 2007). In recent years, concern about this food borne pathogen has increased mainly because of the frequent isolation of antimicrobial resistant strains in humans and animals (Van Looveren *et al.*, 2001; Snelling *et al.*, 2005;) in both developed and developing countries, particularly with regards to the rapid emergence of fluoroquinolone-resistant and multidrug resistant (MDR) *Campylobacter*. According to Hakanen *et al.* (2003) MDR can be significantly associated with resistance to ciprofloxacin, among the few drugs of choice for antibiotic therapy of

campylobacteriosis in humans. MDR is problematic when associated with resistance to ciprofloxacin because of the extremely limited range of treatment options in that situation. Ronner *et al*. (2004) reported that more than 94% of campylobacters isolated from Finnish travelers to Asia and southern Europe showed resistance to one or more antibiotics.

The aims of the present study were to determine the prevalence of *Campylobacter* spp. in live chickens sold at wet markets for slaughter using cloacal and caecal swab samples and to determine the MDR profiles of *Campylobacter* species isolated.

MATERIALS AND METHODS

Collection of samples

A total of 90 live chickens were collected from six wet markets in six areas in Selangor. In all the wet markets, there were a number of stalls selling live chickens and dressed chicken carcasses. The customers may opt to purchase dressed chickens displayed on the stall counters or choose a live chicken and has it freshly slaughtered and dressed. Before slaughter, a cloacal swab was taken from each bird and placed individually in a sterile bottle. The bird was then slaughtered and dressed by the stall workers. Upon evisceration, the intestines were separated; the caeca of each sampled bird were carefully removed from the rest of the intestinal tract, placed in a sterile petri dish. The dishes were sealed, transported in a cool box packed with ice to the laboratory and cultured within two to four hours. Samplings were done over a period of three months.

Isolation and identification of *Campylobacter*

In the laboratory, the caecal contents of each bird were gently squeezed out aseptically and the caeca opened using scissors (sterilized using a burning flame), to expose the mucosal surface which were then swabbed. The caecal contents were intended for another study. Moreover, according to Lee *et al.* (1986) and Berry *et al.* (1988), *Campylobacter* were observed to colonize the mucus on the mucosal surface and within the caecal crypts.

Each cloacal and caecal swab were streaked directly onto separate Campylobacter Blood Free Selective Agar (Oxoid) supplemented with CCDA Campylobacter Selective Supplement (Oxoid). The plates were incubated at 42°C for 48 h, under microaerophilic condition which was generated by using an anaerobic jar containing a gas generating pack (CampyPak™ EZ, BD). Plates were examined for colonies typical of *Campylobacter*. Suspected colonies were then examined for oxidase positive, gram negative, slender, spiral curved rods with typical corkscrew, twirling and rapid darting movements. Two to three colonies presumptively identified as *Campylobacter* colonies were transferred onto Columbia Blood Agar (Oxoid) plate supplemented with defribinated sheep blood (Oxoid), then incubated at 37°C for 24 h under microaerobic condition.

Identification to species level was subsequently performed on colonies isolated from the blood agar plates using an identification kit, MAST ID™ Camp Identification System (Mast Diagnostics) which consisted of hippurate hydrolysis, indoxyl acetate hydrolysis and urease tests. These three tests differentiate *Campylobacter* isolates into four species, namely *C. jejuni*, *C. coli*, *Campylobacter lari* and *Campylobacter upsaliensis*.

Antibiotic susceptibility test

Antibiotic susceptibility test for *Campylobacter* isolates was performed using the agar disk diffusion method as described by the National Committee for Clinical Laboratory Standards (2002) (now known as Clinical and Laboratory Standard Institute, CLSI), using *E. coli* ATCC 25922 and *C. jejuni* ATCC 33560 as quality control purposes. A number of studies have reported disk diffusion method to be reliable and easy tool for monitoring the prevalence of resistance in *Campylobacter* in poultry and a suitable alternative method to agar-based MIC methods (Frediani-Wolf & Stephen, 2003; Ronner *et al*., 2004; Miflin *et al.*, 2007).

The antibiotic disks (Oxoid) used in this study were as follows: gentamicin, CN (10µg), enrofloxacin, ENR (5µg), chloramphenicol, C (30µg), erythromycin, E (15µg), tetracycline, TE (3µg), and cephalotin, KF (30). Mueller Hinton Agar (Oxoid) was supplemented with 5% defribinated sheep blood and *Campylobacter* Growth Supplement (Oxoid). The plates were incubated at 37°C for 48 h under microaerophilic atmosphere. The zone diameter was then measured and interpretive criteria as resistant, intermediate or considered to be susceptible were as those specified by NCCLS (2002). *Campylobacter* isolates classified as intermediate were considered to be susceptible to antibiotics.

A number of works (Twaites & Frost, 1997; Van Looveren *et al.*, 2001; Miflin *et al.*, 2007;) defined MDR strains as resistant to four or more antimicrobial groups whereas Hakanen *et al.* (2003) reported as resistant to three or more antibiotics as MDR. In this study, resistance to three or more and four or more antibiotics was reported.

Statistical analysis

Statistical analyses were performed using Fisher's exact two-tailed test using SPSS 17 software. A P- value of <0.05 value was used for statistical significance.

RESULTS

Prevalence of *Campylobacter* spp. in chickens

Of the 90 chickens sampled, 68 (75.6%) were found to be positive for *Campylobacter* spp. Fifty-three of 90 (58.9%) of *Campylobacter* spp. were isolated from caecal swabs compared to 34 of 90 (37.8%) from cloacal swabs (Table 1). Nineteen of the chickens were positive for both caecal and cloacal swabs. Although a higher number of *Campylobacter* was isolated from caeca, the isolation rate did not differ significantly ($p<0.05$) from cloaca. The majority of the isolates from caeca were *C. coli* compared to cloaca which had more *C. jejuni*, again the difference was not significant ($p<0.05$). *Campylobacter lari* was not isolated.

Patterns of antibiotic resistance in *Campylobacter* isolates

The resistance of *Campylobacter* spp. isolates to six antibiotics is presented in Table 2. None of the isolates showed resistance to chloramphenicol (0%). Resistance to cephalothin was observed in 95.5% of isolates; this was followed by resistance to tetracycline in 80.8%, erythromycin in 51.4%, enrofloxacin in 42.4% and gentamicin in 24.4% of isolates. Of the *C. jejuni* isolates, 78.4% were resistant to three or more antibiotics and 54.0% resistant to four or more antibiotics whereas 41.9% and 12.9 % of *C. coli* were resistant to three or more and to four or more antibiotics respectively. Overall, the MDR in the *Campylobacter* isolated was 61.8% (resistant to three or more antibiotics) or 35.3% (resistant to four or more antibiotics). The observed proportional prevalence of MDR was higher than that reported in other studies.

Campylobacter jejuni showed nine resistance profiles with gentamicin-eryhtromycin-enrofloxacin-cephalothin-tetracycline (32.4%) combination the most common MDR profile while *C. coli* showed six profiles, with cephalothin-tetracycline (32.2%) the most common profile (Table 3).

DISCUSSION

A number of studies on the occurrence of *Campylobacter* in both broiler chickens in the farms and village chickens have been

Table 1. Occurrence of *Campylobacter* species in chickens

Sites	No. of samples	No. positives (%)	No. of *C. jejuni* (%)	No. of *C. coli* (%)
Caeca	87	53 (60.9%)	18 (33.9%)	35 (66.0%)
Cloaca	87	34 (39.1%)	23 (67.6%)	11 (32.4%)

Table 2. Resistance of *Campylobacter* isolates to numbers and types of antibiotics

No. abs*	Percentages (%) of isolates resistant to no. of antibiotics					
	1	2	3	4	5	6
C. jejuni (n=37)	10.8	8.3	24.3	21.6	32.4	0
C. coli (n=31)	3.2	48.3	29.0	9.6	3.2	0
Types of abs	Percentages (%) of isolates resistant to types of antibiotics					
	C	CN	E	ENR	KF	TE
C. jejuni (n=37)	0	51.3	45.9	61.6	97.3	86.5
C. coli (n=31)	0	3.2	58.1	12.9	93.5	74.2

*abs – antibiotics: C, Chloramphenicol; CN, Gentamicin; E, Erythromycin; ENR, Enrofloxacin; KF, Cephalothin; TE, Tetracycline

Table 3. Resistance pattern profiles of *C. jejuni* and *C. coli*

Resistance pattern profiles	No. of *C. jejuni* isolates (%) (n = 37)	No. of *C. coli* isolates (%) (n = 31)
Resistance to six antibiotics	0	0
Resistance to five antibiotics		
TE-KF-CN-ENR-E	12 (32.4)	1 (3.2)
Resistance to four antibiotics		
TE-KF-CN-ENR	4 (10.8)	0
TE-KF-CN-E	2 (5.4)	0
TE-KF-ENR-E	2 (5.4)	3 (9.6)
Resistance to three antibiotics		
TE-KF-CN	7 (18.9)	0
TE-KF-ENR	1 (2.7)	0
TE-KF-E	1 (2.7)	9 (29.0)
Resistance to two antibiotics		
TE-KF	3 (8.1)	10 (32.2)
KF-E	0	5 (16.1)
Resistance to one antibiotic		
KF	4 (12.9)	1 (3.2)

*abs – antibiotics: C, Chloramphenicol; CN, Gentamicin; E, Erythromycin; ENR, Enrofloxacin; KF, Cephalothin; TE, Tetracycline

previously carried out in Malaysia. The studies reported the occurrence of *Campylobacter* in broiler chickens at farm level was 72.6%, ranging from 46.3% to 93.3% and in village chickens at 81.9% (Saleha, 2002; Pezzotti *et al.*, 2003). This study showed that *Campylobacter* is still prevalent in broiler chickens in Malaysia as in other countries worldwide. In this study the isolation of *C. coli* was slightly more frequent than *C. jejuni* – this differs from most studies in other countries, except that of

Pezzotti *et al.* (2003) who had isolated a higher number of *C. coli* from broiler chickens, at 55.6% as compared to 44.4% *C. jejuni*. Also, in this present study and other studies, *Campylobacter* were recovered more frequently from caeca swabs than from caecal swabs, however, the difference was not significant.

Several authors, including Andersen *et al.* (2006), Taremi *et al.* (2006) and Pezzotti *et al.* (2003), had used disk diffusion method to study antibiotic resistance among campylobacters isolated from poultry. Cokal *et al.* (2009) found high correlation between agar disk diffusion method and E-test and Luangtongkum *et al.* (2007) demonstrated a high-level correlation between agar dilution and agar disk diffusion method. Although the MIC method is preferred, WHO has recommended use of disk diffusion in limited resource situations.

The percentage of the campylobacters isolated in this study which were resistant to three or more antibiotics was almost three times higher than reported by Hakanen *et al.* (2003) at 22% in Finland. Bester & Essack (2008) reported 23% of broiler chickens had *Campylobacter* resistant to four or more antibiotics which was lower than our finding.

None of the *Campylobacter* isolated in this study was resistant to chloramphenicol compared to a previous study in Malaysia in 2002 in which 22.4% of campylobacters isolated from chickens were found resistant to chloramphenicol (Saleha, 2002). In Sweden too, the resistance to chloramphenicol was 0% (Ronner *et al.*, 2004) and very much less (2.8%) in Iran (Taremi *et al.*, 2006). The resistance to erythromycin was considered frequent, which was also reported by Ge *et al.* (2003) and Van Looveren *et al.* (2001). Pezzotti *et al.* (2003) found more frequent erythromycin resistance in *C. coli* (45.0%) compared to *C. jejuni* (3.1%), which was also shown by *C. coli* isolated from pigs (42.6% vs 0%) and cattle (28.6% vs 8.3%). In contrast, there was no erythromycin-resistant *Campylobacter* strain reported in Iran and Sweden (Ronner *et al.*, 2004; Cokal *et al.*, 2009). Resistance to gentamicin was less, similar to the studies by Wilson (2003) and Pezzotti *et al.* (2003).

Resistance to enrofloxacin too was rather frequent in this study. Wilson (2003) showed resistance to floroquinolones (FQs) in campylobacters can be rapidly induced by mutations in the DNA gyrase and topoisomerase IV genes, supported by studies which found frequent resistance to enrofloxacin and/or ciprofloxacin, ranged from 42.2% to 77% (Van Looveren *et al.*, 2001; Pezzotti *et al.*, 2003; Taremi *et al.*, 2006). It is most likely due to these antibiotics are widely used in poultry and partly due to FQ-resistant *Campylobacter* strains being biologically fitter in the chickens and outcompete majority of FQ-susceptible strains (Snelling *et al.*, 2005).

Campylobacters are usually intrinsically resistant to cephalosporin including cephalothin, with rates up to 100% (Pezzotti *et al.*, 2003). The present study showed 93.5-97.2% of campylobacters resistant to cephalothin similar to that of Suzuki & Yamamoto (2009) at 95.6-96.0%. The resistance to tetracycline was very frequent, similarly reported by other authors (Lee *et al.*, 1994; Van Looveren *et al.*, 2001; Taremi *et al.*, 2006; Bester & Essack, 2008). Tetracycline can survive longer in the environment than do other antibiotics, thus could cause bacteria to become resistant (Frost, 1991). In Sweden and other Scandinavian countries, resistance to antibiotics is very less frequent or none at all including to tetracycline and FQs; farmers in these countries are prohibited from using antibiotics in animal feed as well as restriction are imposed in prescriptions of antibiotics by doctors to humans (Ronner *et al.*, 2004).

It is known that antimicrobial resistance differs between *Campylobacter* species; therefore, it is appropriate to report resistance in *Campylobacter* by species; for example, 75% *C. coli* were resistant to ciprofloxacin compared to 42.2- 45.3% of *C. jejuni* (Pezzotti *et al.*, 2003) with similar findings in other studies (Van Looveren *et al.*, 2001; Taremi *et al.*, 2006). Thwaites & Frost (1997) reported that MDR was more common in *C. coli* (20%) and *C. lari* (60%) compared to *C. jejuni* (11%). Idris *et al.* (2006) worked on resistant *C. coli* and found the organisms

can occur in multiple levels of an integrated poultry system, from a flock of commercial broiler breeders (treated with poultry FQs) to day-old chicks in the hatchery to broiler chickens (progeny of the studied breeder flock) in the farms.

This study showed antibiotic resistant-campylobacters were common among chickens in Selangor which can find their way into the food chain. Chai *et al.* (2007) had suggested that the handling and packaging of *Campylobacter*-contaminated chicken meat at supermarkets could lead to cross-contamination of the organisms onto salad vegetables which usually are eaten raw and therefore could pose a health hazard. In the wet markets visited, some chicken stalls were located near stalls selling vegetables.

Both erythromycin and FQs are drugs of choice for treatment in humans for systemic infections or in severe or long-lasting cases of enteritis (Taremi *et al.*, 2006). In the present study, 60% of erythromycin-resistant *Campylobacter* isolates were enrofloxacin resistant and 71% of these erythromycin-resistant isolates were MDR. With frequent occurrence of resistant *Campylobacter* to these antibiotics in Malaysia, the choice of such drugs could be considered as "guarded"; thus, antibiotic susceptibility test is required before treatment is initiated in *Campylobacter*-infected patients. The presence of MDR campylobacters, which was also reported to be common in southern Europe and Thailand, could be due to the widespread use of antibiotics in chickens, particularly in the feed (Ronner *et al.*, 2004). Thus, there is an urgent need for prudent use of antibiotics in poultry production to reduce the development and spread of MDR *Campylobacter* which must be diligently monitored.

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