Seasonality and antibiotic resistance of Campylobacter *in Turkish chicken meat*

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Keywords
Antimicrobial resistance, Chicken meat, *Campylobacter coli*, *Campylobacter jejuni*, Seasonal prevalence.

Summary
This study investigated the seasonal prevalence and the antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter coli* in 264 samples of chicken meat. The samples encompassed wings (n=88), breasts (n=79) and thighs (n=97) and were purchased from different butcheries and markets in Elazig province, in Eastern Turkey, between December 2009 and November 2010. The meat samples were tested for *Campylobacter* presence and the collected isolates were identified as *Campylobacter jejuni* and *Campylobacter coli* using polymerase chain reaction (PCR). Resistance rates to 7 antimicrobials were investigated by the disk diffusion method. *Campylobacter jejuni* was found at a higher prevalence (41.7%) than *C. coli* (14.4%); *C. jejuni* was isolated most frequently from breast samples (53.2%) than from thighs (40.2%) and wings (32.9%) samples. The prevalence of *C. jejuni* and *C. coli* peaked during the Summer (June-August), with the highest peak occurring in July (77.3%). The lowest prevalence (30%) was detected in February. The prevalence in the Summer (June-August) was significantly higher (71.2%) than the one reported during the Winter (December-February) (39.4%, $P < 0.05$). The highest resistance rate among *C. jejuni* isolates was observed to tetracycline (38.2%), nalidixic acid (29.1%), and ciprofloxacin (24.5%). *Campylobacter coli* also showed a high resistance to these antibiotics, although in slightly different proportions: tetracycline (42.1%), ciprofloxacin (31.6%), and nalidixic acid (26.3%). None of the *C. jejuni* or *C. coli* isolates was resistant to gentamicin.

Variazioni stagionali e resistenza ad antibiotici del Campylobacter *in campioni di carne di pollame in Turchia*

Parole chiave
Campylobacter coli, *Campylobacter jejuni*, Carne di pollo, Prevalenza stagionale, Resistenza antimicrobica.

Riassunto
Nel presente studio si analizza la variazione stagionale della prevalenza e la resistenza antimicrobica di *Campylobacter jejuni* e *Campylobacter coli* in 264 campioni di carne di pollo, mensilmente prelevati dai mercati e dalle macellerie della della provincia di Elazig, nella Turchia orientale tra dicembre 2009 e novembre 2010. I campioni di ali (n=88), petti (n=79) e cosce (n=97) sono stati testati per verificare la presenza di *Campylobacter*, mediante polymerase chain reaction (PCR). Sono stati identificati isolati di *C. jejuni* e *C. coli* ed è stato analizzato il livello di resistenza a 7 antimicrobici mediante disco-diffusione. *Campylobacter jejuni* è stato rilevato in una percentuale più alta (41.7%) rispetto al *C. coli* (14.4%); *C. jejuni* è stato isolato più frequentemente nei campioni di petti di pollo (53.2%) che nei campioni prelevati dalle cosce (40.2%) e dalle ali (32.9%). Inoltre, è stato riscontrato che prevalenza di *C. jejuni* e *C. coli* raggiunge livelli superiori durante i mesi estivi (giugno-agosto), con un picco nel mese di luglio (77.3%), mentre la percentuale più bassa di prevalenza è stata registrata nel mese di febbraio (30%). La prevalenza ha livelli più alti nei mesi estivi (71.2%) rispetto a quelli invernali (39.4%, $P < 0.05$). Tra gli isolati di *C. jejuni* il tasso più alto di resistenza è stato osservato per la tetraciclina (38.2%), seguito da acido nalidixico (29.1%), e ciprofloxacina (24.5%). Anche *C. coli* ha mostrato un'alta resistenza agli stessi antibiotici, seppure con diverse percentuali: tetraciclina (42.1%), ciprofloxacina (31.6%), e acido nalidixico (26.3%). Gli isolati di *C. jejuni* e *C. coli* non hanno mostrato resistenza alla gentamicina.

Introduction

Campylobacter is the major bacterial cause of human gastroenteritis worldwide (Coker *et al.* 2002, Friedman et al. 2000, World Health Organization 2010). *Campylobacter jejuni* and *Campylobacter coli* account for approximately 90% and 10% of human infections, respectively (Friedman *et al.* 2000). Other *Campylobacter* species are occasionally isolated from clinical cases but at much lower proportions (Frost *et al.* 1998, Leatherbarrow *et al.* 2004). It has been proved that handling of raw poultry and consuming undercooked chicken meat are important sources of campylobacterosis in humans (Dufrenne *et al.* 2001, Michaud *et al.* 2004). The incidence of campylobacteriosis in humans generally peaks during the Summer (Tauxe 1992). In Turkey, the incidence of campylobacteriosis in humans has peaked during March, May, July and August suggesting seasonality (Gurol *et al.* 2013).

Campylobacter jejuni and *C. coli* are phylogenetically closely related, the differentiation between the two species has been performed using biochemical assays, such as the hippurate hydrolysis test (Dedieu *et al.* 2004). However, speciation using biochemical techniques sometimes disagrees with genetic based methods such as the polymerase chain reaction (PCR) (Dedieu *et al.* 2004, Rönner and Lindmark 2007). The identification by PCR is the most common and reliable method to rapidly differentiate between *Campylobacter* species (Bolton *et al.* 2002, Zaidi *et al.* 2012). Different PCR strategies including various genetic targets such as *ceuE* (Gonzalez *et al.* 1997), aspartokinase (asp), hippuricase gene (*hipO*) (Lawson *et al.* 1998), *cadF* (Englen and Fedorka-Cray 2002), and 16S rRNA (Bang *et al.* 2002) have been used to identify *C. coli* and *C. jejuni* and *Campylobacter* species.

Campylobacteriosis is often self-limiting and generally does not require antimicrobial treatment (Allos 2001, Corcoran *et al.* 2006), except in immunocompromised patients such as the very young or elderly people (Bardon *et al.* 2011, Wieczorek *et al.* 2012). Recently, the number of *Campylobacter* strains resistant to fluoroquinolones has increased worldwide (Alfredson and Korolic 2007, European Food Safety Authority 2012b, Quinn *et al.* 2007, Wieczorek *et al.* 2012). In Turkey, the increasing of antibiotic resistance has become a major public health problem (Yucel and Erguler 2008). Of human *Campylobacter* isolates in Turkey, 59% and 7% were resistant to quinolone and erythromycin, respectively (Gurol *et al.* 2013, Ongen *et al.* 2007), the percentage of resistance varying depending on the antibiotics tested. It has been reported that 79.5% of *Campylobacter* isolates were resistant to nalidixic acid, 75.6% to levofloxacin, 73.9% to ciprofloxacin, 24% to tetracycline and 6.3% to erythromycin (Kayman *et al.* 2013).

The aim of this study was to analyse the seasonal differences in the prevalence of *C. jejuni* and *C. coli* in retail chicken meats collected in Elazig province in Eastern Turkey and to measure the antibiotic resistance of the isolates.

Materials and methods

Collection of chicken samples

A total of 264 samples of chicken meat (with skin) were collected monthly between December 2009 and November 2010 from different butchers' shops and markets in the Elazig province, in Eastern Turkey. Samples included wings (n=88), breasts (n=79) and thighs (n=97). Meat samples were individually bagged, and immediately transported to the laboratory on ice, refrigerated and processed within 24 hours from collection.

Culture of *Campylobacter* spp.

The detection of *Campylobacter* spp. was conducted according to the ISO 10272-1:2006 standards[1]. Each chicken piece (e.g. a single wing, thigh or breast) was analysed individually. Slices were cut aseptically from each pack. For all samples, 25 gr were added to 225 mL of supplemented Bolton Broth (Oxoid CM 983, SR 183, SR 48, Basingstoke, UK) and incubated under microaerophilic conditions obtained by the CampyGen gas-generating kit (Oxoid, Lot: 13L08-C25-14, Basingstoke, UK) at 35°C for 4 hours prior to transfer to 41.5°C for 48 hours. Following enrichment, 10 µl of broth were spread onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Oxoid CM 739, SR 155, Basingstoke, UK) and incubated at 41.5 °C for 24-48 hours microaerobically. At the end of the incubation period, the plates were evaluated for the presence of suspected *Campylobacter* colonies. Five presumptive colonies were selected, plated on sheep blood agar and incubated at 41.5°C microaerobically for 24-48 hours.

The suspect *Campylobacter* colonies were accepted as *C. jejuni* and *C. coli* by examination of morphology, gram staining and biochemical tests for oxidase, catalase, hippurate and indoxyl acetate hydrolysis[2].

[1] International Organization for Standardization (ISO). 2006. Microbiology of food and animal feeding stuffs – horizontal method for detection and enumeration of thermotolerant *Campylobacter* spp. Part 1: detection method. ISO 10272-1:2006.

[2] Health Protection Agency (HPA). 2005. Detection of *Campylobacter* species. National Standard Method F 21, Issue 2. http://www.hemltd.ru/export/sites/HemLtd/publications/sections/Normativ/foreign/Infections/medicine/NHS019/article.pdf.

The isolates were preserved in brain heart infusion broth (BHIB) with 15% (v/v) glycerol at – 80°C for subsequent analysis.

DNA extraction and PCR

The isolation of genomic DNA from *C. jejuni* and *C. coli* isolates was performed using the QIAamp DNA mini kit (Qiagen, Hidelberg, Germany) as instructed by the manufacturer. The amplification was conducted in a reaction mixture containing 2 X PCR Master Mix kit (# K01071, Fermentas, Lithuania) using *C. jejuni* and *C. coli* specific primers and thermal cycling conditions as previously established by Misawa and colleagues (Misawa *et al.* 2002). Ten microliters of the amplified PCR products were mixed with 2 µl of loading buffer and electrophoresed on a 1.5% agarose gel and visualized using a UV transilluminator. The PCR amplification product of the putative oxidoreductase and aspartokinase genes provided band sizes of 159 bp and 502 bp for *C. jejuni* and *C. coli* isolates, respectively. Characterized *C. jejuni* and *C. coli* isolates previously recovered from chicken faecal samples were used as positive controls (Ozbey *et al.* 2012) and distilled water was used as negative control in each PCR reaction.

Antimicrobial resistance testing

Antimicrobial susceptibility was conducted using the disc diffusion method on Muller Hinton agar (MHA) (Oxoid CM 337, Basingstoke, UK) containing 5% defibrinated sheep blood as described by the National Committee for Clinical Laboratory Standards[3]. Using antimicrobial susceptibility testing discs, 110 *C. jejuni* and 38 *C. coli* isolates were investigated for resistance to antibiotics including ciprofloxacin (5 µg), nalidixic acid (30 µg), enrofloxacin (5 µg), tetracycline (30 µg), gentamicin (10 µg), erythromycin (15 µg), and chloramphenicol (30 µg) (Oxoid, Basingstoke, UK). The inhibition zone sizes around the discs were measured using callipers and interpreted as resistant (R) or susceptible (S) according to National Committee for Clinical Laboratory Standards.

Statistical analysis

Both Fisher's exact test and the Chi squared test (χ^2) were used to compare chicken pieces (wings, thighs and breasts) for *C. jejuni* and *C. coli*; for the seasonal differences in the prevalence of *Campylobacter*, a *P* value of < 0.05 was accepted as significant.

[3] National Committee for Clinical Laboratory Standards (NCCLS). 2003: Performance standards for antimicrobial disk susceptibility tests. 8th ed. Approved standard NCCLS document M2-A8. NCCLS, Wayne, PA.

Results

Campylobacter jejuni and *C. coli* were obtained from 110 (41.7%) and 38 (14.4%) of the 264 chicken samples, respectively. The percentage of chicken testing positive for *C. jejuni* differed among the chicken product types and was highest on breast samples (53.2%), followed by thighs (40.2%) and lowest in wings (32.9%). Using the Fisher's exact test, no significant difference was noted between chicken parts. *Campylobacter coli* differed by having the highest number of positive samples on thigh pieces (23.7%), followed by breasts (11.4%) and, similarly to *C. jejuni*, was lowest in wings (6.8%). Again, chicken pieces did not significantly differ for *C. coli* prevalence.

Table I shows the seasonal and monthly changes in the percentage of *C. jejuni* and *C. coli* isolated from raw chicken. *Campylobacter* spp. (*C. jejuni* and *C. coli*) was detected throughout the period of this study, with the most frequent isolation of *Campylobacter* spp. observed in July (77.3%), followed by June (70%) and August (66.7%). The lowest percentage prevalence was detected in February (30%). Using the Fisher's exact test, the percentage of chicken samples testing positive during the Summer (June-August) (71.2%) was significantly higher than the percentage of the samples testing positive during the Winter season (December-February) (39.4%) ($P < 0.05$).

The percentage of the antibiotic resistance of *C. jejuni* and *C. coli* isolates are summarized in Table II. The antibiotic with the highest number of

Table I. *The seasonal changes in the prevalence of* C. jejuni *and* C. coli *from the samples of retail poultry collected between December 2009 and November 2010 in the Elazig province, Eastern Turkey.*

Seasons	Months	Number of samples tested (n)	*C. jejuni* n (%)	*C. coli* n (%)	Total n (%)
Winter	December	24	8 (33.3)	4 (16.7)	12 (50)
	January	22	6 (27.3)	2 (9.1)	8 (36.4)
	February	20	5 (25)	1 (5)	6 (30)
Spring	March	21	10 (47.6)	3 (14.3)	13 (61.9)
	April	20	10 (50)	1 (5)	11 (55)
	May	22	11 (50)	3 (13.6)	14 (63.6)
Summer	June	20	13 (65)	1 (5)	14 (70)
	July	22	16 (72.7)	1 (4.6)	17 (77.3)
	August	24	13 (54.2)	3 (12.5)	16 (66.7)
Autumn	September	22	9 (40.9)	5 (22.7)	14 (63.6)
	October	25	5 (20)	8 (32)	13 (52)
	November	22	4 (18.2)	6 (27.3)	10 (45.5)
Total		**264**	**110 (41.7)**	**38 (14.4)**	**148 (56.1)**

Table II. *Antimicrobial resistance rates of* C. jejuni *and* C. coli *isolated from the samples of retail poultry meat collected between December 2009 and November 2010 in the Elazig province, Eastern Turkey.*

Antibiotics	Number of resistant isolates (%)		
	*C. jejuni*n (110)	*C. coli* n (38)	Total n (148)
Ciprofloxacin	27 (24.5)	12 (31.6)	39 (26.4)
Nalidixic acid	32 (29.1)	10 (26.3)	42 (28.4)
Tetracycline	42 (38.2)	16 (42.1)	58 (39.2)
Chloramphenicol	3 (2.7)	2 (5.3)	5 (3.4)
Enrofloxacin	21 (19.1)	5 (13.2)	26 (17.6)
Erythromycin	1 (0.9)	0	1 (0.7)
Gentamicin	0	0	0

resistant *C. jejuni* and *C. coli* isolates was tetracycline (38.2% and 42.1%), followed by nalidixic acid (29.1% and 26.3%) and ciprofloxacin (24.5% and 31.6%). *Campylobacter jejuni* showed a low resistance to chloramphenicol (2.7%) and erythromycin (0.9%). None of the *C. coli* isolates were found to be resistant to erythromycin. In addition, all of the isolates (*C. jejuni* and *C. coli*) were susceptible to gentamicin.

Discussion

Prior to this study, data on the seasonal changes in the prevalence of *C. jejuni* and *C. coli* in raw chicken meats and the antibiotic resistance of the isolates from Eastern Turkey were limited.

Campylobacter prevalence in chicken meats varies between areas and countries. In this respect it is noteworthy that *Campylobacter* prevalence been reported to be 90% in both South Australia and New South Wales (Pointon *et al.* 2008), 32% in South Africa (van Nierop *et al.* 2005), 29% in Belgium (Wieczorek *et al.* 2012), approximately 50% in Ireland (Whyte *et al.* 2004, Wilson 2002), 76% in France (Denis *et al.* 2001), 80% in Holland (Dufrenne *et al.* 2001), and 83.8% in Turkey (Savasci and Ozdemir 2006). The overall *Campylobacter* prevalence (56.1%) reported in this study is consistent with some international and national studies.

This study also confirmed that *C. jejuni* is the predominant species in chicken meat in Turkey. This data agrees with the other findings focusing on Turkey and other countries as well (European Food Safety Authority 2012a, Bardonet *et al.* 2011, Bostan *et al.* 2009, Kang *et al.* 2006, Yucel and Erguler 2008). Conversely, other authors have reported that *C. coli* was identified at a higher percentage than *C. jejuni* from raw poultry meat (Kudirkienė *et al.* 2013, Wieczorek *et al.* 2012). However, research performed on chicken meats in Turkey indicated that the prevalence of *C. jejuni* varied between 65.9% (149/226) (Bostan *et al.* 2009) and 74.8% (95/127) (Savasci and Ozdemir 2006). The current study showed a relatively low prevalence of *C. jejuni* at 41.7% (110/264) and *C. coli* at 14.4% (38/264) when compared to other studies. The prevalence of *C. coli* in Turkey has been reported to be 17% (51/300) and 25.2% (57/226) (Bostan *et al.* 2009, Yucel and Erguler 2008). The variability in the *Campylobacter* prevalence in retail chicken meat may be due to geographic differences, variation in hygiene practices during slaughtering and processing, and variation in the methods used to culture and identify *Campylobacter* species.

It has previously been reported that the concentration and prevalence of *Campylobacter* on chicken meat differs significantly between different producers (Kudirkienė *et al.* 2013). Similar to this study, in Turkey chicken breast samples were more contaminated with *C. jejuni* at 45.7% (21/46) and 86% (37/43) than the other parts of the bird (Bostan *et al.* 2009, Savasci and Ozdemir 2006). This may be explained with the attachment characteristics of the bacteria and the larger surface and follicule structure on breast meat (Kotula and Pandya 1995, Savasci and Ozdemir 2006).

The highest isolation rate of *Campylobacter* from chicken meat was detected during the Summer (71.2%), while the lowest rate was during the Winter (39.4%), this is consistent with literature that reports a peak in the isolation of *Campylobacter* from chicken meat during the warmer months (Peterson *et al.* 2001, Willis and Murray 1997). A study conducted in Iran also reported that the prevalence of *Campylobacter* spp. in chicken meat was the highest during July (87.5%), followed by June (81.8%), September (76.7%) and August (68.8%). Similarly, the lowest prevalence was recorded in February (20.7%) (Rahimi and Saljoughian Esfahani 2010). Seasonal variability in the proportion of species isolated has also been reported where *C. jejuni* was detected more often during the warmer months (April-October), and *C. coli* was more common during the colder periods (November-March) (Kudirkienė *et al.* 2010). This data support the results of the current research. In contrast, only 1 study conducted in Turkey reported no seasonal variability in the *Campylobacter* detection rates from chicken (Bostan *et al.* 2009).

The susceptibility of *C. jejuni* and *C. coli* to 7 antimicrobials showed that the highest resistance was to tetracycline, nalidixic acid and ciprofloxacin; this may be due to the widespread and overuse of antibiotics in animal agriculture. The European Commission discourages the use of antibiotics as a preventative measure and encourages prudent use of antimicrobials by enhancing on farm biosecurity

practices to reduce zoonotic and symptomatic infections in poultry production[4]. However, there are considerable differences in legislation regulating the use of antibiotics in poultry production across Europe[5]. This may partially account for the large disparities in antimicrobial resistance among European countries, with some having stricter regulations than others[6]. According to the EFSA report (European Food Safety Authority 2012b), resistance to nalidixic acid and ciprofloxacin is common among *Campylobacter* isolates from chicken meat in many European States, but it is low in Nordic countries (Andersen *et al.* 2006, Bardon *et al.* 2011).

Campylobacter jejuni isolates from chickens had a ciprofloxacin resistance of 19% in the USA (Gupta *et al.* 2004) and 14.9% in Europe (Bywater *et al.* 2004). It is noteworthy that the resistance to ciprofloxacin tended to be much higher in Turkey at 25% and 74.2% (Cokal *et al.* 2004, Yucel and Erguler 2008). In Europe, 39.6% of *C. coli* isolates from chicken were resistant to ciprofloxacin (Bywater *et al.* 2004, Dufrenne *et al.* 2001). Again, this tended to be much higher in Turkey, with *C. coli* ciprofloxacin resistance being at 51% and 78.1% (Savasan *et al.* 2004, Yucel and Erguler 2008). In the current study, ciprofloxacin resistant *C. jejuni* at 24.5% is one of the highest percentages reported in Europe and the lowest percentage reported in Turkey. Furthermore, the prevalence of ciprofloxacin resistant *C. coli* at 31.6% is lower than the previously reported values in both Turkey and Europe.

The tetracycline resistance was 34.5% in European countries (Bywater *et al.* 2004) and 43% in the USA (Gupta *et al.* 2004). Two studies conducted in Turkey, reported tetracycline resistance to be 42% and 76.3% for *C. jejuni* and 58.1% and 55.2% for *C. coli* isolated from broilers (Cokal *et al.* 2009, Yildirim *et al.* 2005). Another study conducted in Turkey showed lower tetracycline resistance at 15.3% and 24.2% for *C. jejuni* and *C. coli*, respectively (Erdeger and Diker 1995). In the current study the percentages of *C. jejuni* and *C. coli* isolates resistant to tetracycline were 38.2% and 42.1%, respectively.

Earlier studies reported that the number of *Campylobacter* isolates resistant to macrolides was low and did not exceed 1% (Andersen *et al.* 2006, Wieczorek *et al.* 2012), supporting the present findings. These lower resistance rates are probably due to the fact that macrolides are not used in veterinary medicine in Turkey (Yucel and Erguler 2008). Contrary to our results, studies carried out in Belgium and in the Netherlands showed a relatively high resistance to erythromycin: in Belgium, 4% of *C. jejuni* and 18% of *C. coli* isolates were resistant to erythromycin; whereas, in the Netherlands resistance was observed in 39% of the *Campylobacter* isolates (European Food Safety Authority 2012b). Similar to the current study no gentamicin resistance was exhibited in any other study (Rahimi *et al.* 2010, Wieczorek *et al.* 2012).

Education to raise consumers' awareness and highlighting the methods for the safe preparation of chicken is needed to reduce the human incidence of *Campylobacter*. Intervention strategies at farm and during processing can also aid in the reduction of human *Campylobacter* infections.

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