ORIGINAL ARTICLE

Campylobacter spp. and their Antimicrobial Resistance Patterns in Poultry: An Epidemiological Survey Study in Turkey

Yavuz Cokal[1], Vildan Caner[2], Aysin Sen[3], Cengiz Cetin[3] and Nedim Karagenc[2]

[1] Bandirma Vocational School, Balikesir University, Bandirma, Balikesir, Turkey
[2] Department of Medical Biology, School of Medicine, Pamukkale University, Kinikli, Denizli, Turkey
[3] Department of Microbiology, Faculty of Veterinary Medicine, Uludag University, Gorukle, Bursa, Turkey

Impacts

- A downward trend was observed in the overall prevalence of *Campylobacter* spp. in Turkey.
- The resistance rates to tetracycline and fluoroquinolones were high in *C. jejuni* and *C. coli* isolates.
- There was a great correlation between the disk diffusion method and E-test in both species.

Keywords:
Campylobacter jejuni; *Campylobacter coli*; broiler chickens; prevalence; antimicrobial resistance

Correspondence:
C. Cetin. Department of Microbiology, Faculty of Veterinary Medicine, Uludag University, 16059, Gorukle, Bursa, Turkey.
Tel.: +90 224 2941294;
Fax: +90 224 2941202;
E-mail: cengizc@uludag.edu.tr

Received for publication December 6, 2007

doi: 10.1111/j.1863-2378.2008.01155.x

Summary

The current study aimed at determining the prevalence and the antimicrobial resistance profiles of thermophilic *Campylobacter* spp. infecting broiler chickens. A total of 240 caecal samples from six slaughterhouses were examined for the presence of *Campylobacter* spp. *C. jejuni* was detected in 40.4% (97/240) of the samples and *C. coli* in 12.1% (29/240). The agar disc diffusion method and the E-test were used for testing the antimicrobial susceptibility of *C. jejuni* and *C. coli* isolates. *C. jejuni* isolates were most resistant to nalidixic acid (79.4%) followed by tetracycline (76.3%), ciprofloxacin (74.2%) and enrofloxacin (15.5%). Among the *C. coli* isolates, the frequency of resistance to nalidixic acid and ciprofloxacin was the same at 65.5%. The predominant profiles of multidrug resistance to three or more antimicrobials in *C. jejuni* and *C. coli* were determined as tetracycline/nalidixic acid/ciprofloxacin resistance (48.5%) and tetracycline/nalidixic acid/ciprofloxacin/enrofloxacin resistance (51.7%), respectively. To prevent the transmission of antimicrobial-resistant bacteria of animal origin to humans, it should be noted that high proportions of multidrug resistance were found in both species.

Introduction

During the last few decades, certain enteric bacteria, responsible for the recent foodborne and waterborne epidemics in the world, have re-emerged as human pathogens. Of these pathogens, thermophilic *Campylobacter* spp., including *Campylobacter jejuni* and *Campylobacter coli*, have been recognized as the primary causative agents of bacterial human foodborne gastroenteritis in both industrialized and developing countries (World Health Organization, 2002; Snelling *et al*., 2005). A significant association exists between *Campylobacter* infection in humans and consumption of contaminated poultry products, as revealed by case–control studies (Altekruse *et al*., 1999; Stern *et al*., 2001; Friedman *et al*., 2004). Several epidemiological studies have examined risk factors such as the presence of other animals on the farm, contamination of previous flock as well as vertical transmission for the infection of broiler flocks by *Campylobacter* (Gregory *et al*., 1997; Newell and Fearnley, 2003). In addition, *Campylobacter* biofilms with/without other microorganisms in the water lines of poultry houses could be a continuous source of the contamination (Zimmer *et al*., 2003).

A number of studies observed the high level of antimicrobial resistance in thermophilic *Campylobacter* spp. in poultry and humans before the prohibition of the use of antimicrobials as food additives to promote growth in poultry (Smith *et al*., 1999; Owen and Leeton, 1999; Engberg *et al*., 2001). Data regarding the prevalence and antimicrobial resistance profiles among thermophilic *Campylobacter* isolates of poultry origin in Turkey are limited. Results obtained from previous studies cannot be compared for prevalence rates and resistance profiles because of differences in isolation procedures, sample material used and antimicrobial testing, as well as geographic localization. However, the development of resistance to fluoroquinolones among *Campylobacter* spp. after the introduction of these drugs for the treatment of infections in poultry has been reported and the level of resistance rate was high in broiler chickens (Savasan et al., 2004; Yildirim *et al*., 2005). We aimed at estimating the prevalence of thermophilic *Campylobacter* spp. and determining the current antimicrobial resistance profiles of the isolates from broiler chickens.

Materials and Methods

Sample collection

A total of 240 caecal samples taken from broiler chickens at six different slaughterhouses at Bandirma, Balikesir during a 5-month period between March and July 2006 were studied. This study was conducted after a short period of the prohibition of the use of antimicrobial agents as a feed additive in poultry industries in Turkey. In each slaughterhouse, only one flock was examined and 40 samples were randomly collected. All samples were put into sterile tube, cooled in an icebox and immediately transported to the laboratory for bacteriological culture.

Isolation and identification

Thermophilic *Campylobacter* spp. were isolated from caecal samples using a direct plating method. All samples were homogenized and cultured on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid, Basingstoke, UK) with selective supplement (SR155, Oxoid). All plates were incubated under microaerophilic conditions for 48 h at 42°C. Small, curved, catalase- and oxidase-positive, Gram-negative bacilli were presumed to be *Campylobacter* spp. Identification to species level was subsequently based on the ability of the isolate to hydrolyse sodium hippurate and indoxyl acetate, H_2S production in triple sugar iron agar, and susceptibility to cephalothin (El-Shibiny *et al*., 2005; On and Holmes, 1992). All isolates were transferred to Brucella broth with 7% lysed horse blood and 10% glycerol, and stored at −80°C for antimicrobial susceptibility testing.

Antimicrobial susceptibility testing

All isolates were screened for resistance to amikasin (10 μg), kanamycin (30 μg), streptomycin (10 μg), gentamycin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), ampicillin (10 μg), erythromycin (15 μg), tetracycline (30 μg) and chloramphenicol (30 μg) by the agar disc diffusion method. All cartridges of antimicrobial-containing discs were obtained from Oxoid. A suspension of 0.5 McFarland standard prepared in Mueller-Hinton broth (Oxoid) was inoculated into Mueller-Hinton agar (Oxoid) plates containing 5% (v/v) defibrinated sheep blood and incubated at 37°C for 48 h under microaerophilic conditions (Gaudreau and Gilbert, 1998). Inhibition zones were recorded and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI; formely NCCLS) (National Committee for Clinical Laboratory Standards, 1999).

Minimal inhibitory concentrations (MICs) for tetracycline, nalidixic acid, enrofloxacin and ciprofloxacin were determined by using the E-test (AB Biodisk, Solna, Sweden) in accordance with the recommendations of the manufacturer. Briefly, the bacterial suspension equivalent to 1.0 McFarland standard for each isolate was spread (100 μl) on Petri plates containing Mueller-Hinton agar with 5% defibrinated sheep blood (Biomeriux, Marcy I' Etoile, France). E-test strips were applied on the agar plates after the suspension were absorbed into the agar. Plates were incubated under the same conditions as for the disc diffusion method. The breakpoint values as recommended by the CLSI for veterinary pathogens used to define resistance in the E-test were ≥16, ≥32, ≥4 and ≥4 μg/ml for tetracycline, nalidixic acid, enrofloxacin and ciprofloxacin, respectively (Luber *et al*., 2003). During the testing of the isolates in both methods, *C. jejuni* ATCC 33560, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 were included as the controls in each test batch.

Statistical analysis

The significance of differences in resistance was analysed by using the chi-squared test (SPSS software version 11.5; SPSS Inc., Chicago, IL, USA). A *P*-value of ≤0.05 was considered statistically significant. To measure the correlation and the level of aggrement between the agar disc diffusion and E-test, the kappa statistics were calculated with STATA version 9.0 software (Stata Corp., College Station, TX, USA).

Table 1. *Campylobacter jejuni* and *C. coli* isolated from caecal samples in six different slaughterhouses

	C. jejuni (*n* = 97)		*C. coli* (*n* = 29)	
No. slaughterhouse	*n*	%	*n*	%
1	7	7.2	3	10.3
2	–	–	–	–
3	29	29.9	5	17.2
4	14	14.4	2	6.9
5	26	26.8	10	34.5
6	21	21.6	9	31.0

Results

Prevalence of thermophilic *Campylobacter* spp.

Of the 240 caecal samples processed, 126 (52.5%) were found to be positive for thermophilic *Campylobacter* spp. *C. jejuni* was found in 40.4% (97/240) of the samples and *C. coli* in 12.1% (29/240). The distribution of *Campylobacter* isolates from different slaughterhouses is shown in Table 1.

Antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp.

Ninety-seven *C. jejuni* and 29 *C. coli* isolates were examined for susceptibility to 11 antibiotics using the disc diffusion method. The percentages of isolates showing susceptibility, intermediate susceptibility, and resistance to each antimicrobial agent are presented in Table 2. Of the aminoglicosides included in the panel, sensitivity to amikasin, kanamycin and streptomycin was seen in all *Campylobacter* isolates. The sensitivity profile to gentamycin, the other aminoglicoside in this study, was determined as intermediate in 18 (18.6%) *C. jejuni* isolates while all of the *C. coli* isolates were sensitive to the aminoglicoside. β-lactam resistance was not observed in the isolates, but 44.3% of *C. jejuni* isolates and 51.7% of *C. coli* isolates had an intermediate profile. There was a high resistance to tetracycline in *C. jejuni* (76.3%) and *C. coli* (55.2%) isolates. The highest level of fluoroquinolone resistance of the *C. jejuni* isolates was recorded to ciprofloxacin (74.2%), followed by 15.5% to enrofloxacin. Among the *C. coli* isolates, no significant difference was observed between the rates of resistance to nalidixic acid and ciprofloxacin. But the rates of resistance to enrofloxacin were significantly higher ($\chi^2 = 16.31$; $P < 0.05$) for *C. coli* (51.7%) than *C. jejuni* (15.5%). The predominant profiles of multidrug resistance to three or more antimicrobials in *C. jejuni* and *C. coli* were determined as tetracycline/nalidixic acid/ciprofloxacin resistance (48.5%) and tetracycline/nalidixic acid/ciprofloxacin/enrofloxacin resistance (51.7%), respectively.

The MIC ranges and the proportions of resistance of *C. jejuni* and *C. coli* isolates for the four antimicrobial agents are summarized in Table 3. The MIC range for tetracycline in *C. jejuni* and *C. coli* isolates was 2 to ≥256 μg/ml and 0.125 to ≥256 μg/ml, respectively. One

Table 3. MIC distrubition and resistance levels determined for 126 *Campylobacter* spp. isolates using the E-test

	C. jejuni (*n* = 97)		*C. coli* (*n* = 29)	
Antimicrobial agent	MIC range (μg/ml)	% Resistant (*n*)	MIC range (μg/ml)	% Resistant (*n*)
Tetracycline	2 to ≥256	77.3 (75)	0.125 to ≥256	55.2 (16)
Nalidixic acid	0.125–128	81.4 (79)	1–128	68.9 (20)
Ciprofloxacin	2 to ≥32	74.2 (72)	8 to ≥32	65.5 (19)
Enrofloxacin	0.25–16	15.5 (15)	0.25–32	51.7 (15)

Table 2. Antimicrobial susceptibility patterns of *Campylobacter* spp. identified by the agar disc diffusion method

	No. *C. jejuni* isolates* (*n* = 97)				No. *C. coli* isolates* (*n* = 29)			
Antimicrobial agent	R	I	S	Resistant isolates (%)	R	I	S	Resistant isolates (%)
Ampicillin	–	43	54		–	15	14	–
Gentamycin	–	18	79	–	–	–	29	–
Amikasin	–	–	97	–	–	–	29	–
Kanamycin	–	–	97	–	–	–	29	–
Streptomycin	–	–	97	–	–	–	29	–
Tetracycline	74	23	–	76.3	16	4	9	55.2
Nalidixic acid	77	14	6	79.4	19	10	–	65.5
Ciprofloxacin	72	6	19	74.2	19	10	–	65.5
Enrofloxacin	15	27	55	15.5	15	4	10	51.7
Chloramphenicol	–	–	97	–	–	–	29	–
Erythromycin	–	–	97		–	–	29	–

*Number of susceptible (S), intermediate (I), and resistant (R) *Campylobacter* isolates.

C. jejuni isolate showed intermediate sensitivity in the disc diffusion method but resistance in E-test. A wide range of MICs among antimicrobials used in this study was observed mainly in nalidixic acid for *C. jejuni* isolates. Fifty-one and 25 *C. jejuni* isolates had MIC values of 32 and 64 μg/ml, respectively. In terms of nalidixic acid susceptibility pattern, two *C. jejuni* and one *C. coli* isolates were determined as resistant in E-test and as intermediate in the disc diffusion method. There was a great aggrement on the overall level of resistance, which was determined by both the disc diffusion method and E-test in *C. jejuni* and *C. coli* isolates (kappa = 0.93 and 0.98, respectively).

Discussion

In the present study, *C. jejuni* was isolated from 40.4%, and *C. coli* from 12.1% of the caecal samples of broilers. Species distribution among the positive slaughterhouses showed that all flocks were infected with both species. The overall prevalence of thermophilic *Campylobacter* spp. [52.5% (126/240)] is generally concordant with the results of similar studies carried out in other countries (Jozwiak et al., 2006; Parisi et al., 2007). In Turkey, only limited data exist on the prevalence of thermophilic *Campylobacter* spp. in broilers and *Campylobacter* was recovered at higher prevalences ranging from 91.3% to 95% (Yildiz and Diker, 1992; Yildirim et al., 2005). Therefore, a downward trend was observed in the overall prevalence of *Campylobacter* spp. although differences in the sample type, sampling procedures, isolation methods and season preclude conclusions about the relative prevalences as discussed previously (Newell and Fearnley, 2003). The use of antimicrobial agents as a feed additive in poultry farms was prohibited by the Ministry of Agriculture and Rural Affairs, General Directorate of Protection and Control in January 2006 in Turkey. This study was conducted after a short period of the prohibition. The overall prevalence of thermophilic *Campylobacter* spp. in the current study is lower than that reported from previous studies in Turkey. This dramatic decrease might be explained by the fact that the strategies of hazard analysis and critical control point and/or other hygiene programmes are strictly adapted to the poultry industries in this region.

A national surveillance programme should be applied to assess the resistance pattern of campylobacters in broiler chickens as epidemiological surveillance of antimicrobial resistance is a public health concern with relation to the development of antimicrobial resistance in pathogenic bacteria of food animals. Data concerning antimicrobial resistance in thermophilic *Campylobacter* spp. are limited in Turkey. Yildirim et al. (2005) reported that the resistance rates to tetracycline in *C. jejuni* and *C. coli* from broilers were 42% and 58.1%, respectively. The level of resistance to tetracycline (76.3%) among *C. jejuni* isolates in this study was higher than that of the earlier Turkish report, while the level (55.2%) among *C. coli* isolates was in agreement with the report. Several studies report the high levels of tetracycline in campylobacters from food animals (Saenz *et al*., 2000; Van Looveren *et al*., 2001; Bywater *et al*., 2004), although a decrease to 1% in resistance level in tetracycline after the prohibition of the use of antibiotics as a feed additive has been observed (Rönner *et al*., 2004).

The fluoroquinolones, ciprofloxacin and enrofloxacin, were chosen for antimicrobial susceptibility tests in this study because they are the first- and/or second-line therapeutic agents and/or used for prophylaxis in poultry industries in Turkey. Worldwide, the development of resistance to fluoroquinolones among *Campylobacter* spp. from food animals after the introduction of fluoroquinolones for the above purposes has previously been reported (Saenz *et al*., 2000; Engberg *et al*., 2001; Savasan et al., 2004). Especially, ciprofloxacin resistance frequencies in *C. jejuni* have increased dramatically in the last few decades, approaching 99% in Spain (Van Looveren *et al*., 2001). In contrast, lower levels of ciprofloxacin resistance have been reported in some European countries (Bywater *et al*., 2004), in Ireland (Corcoran *et al*., 2006), and Canada (Guevremont *et al*., 2006). In the current study, the level of ciprofloxacin resistance in *C. jejuni* was 74.2%, similar to the level of 70.6% of the Turkish study by Savasan et al. (2004). But a decrease in ciprofloxacine resistance (65.5%) for *C. coli* was observed when compared with the previous study, which reported the level of ciprofloxacin resistance to be 78.1% in *C. coli* isolates in broilers (Savasan et al., 2004). This study highlights that the overall level of resistance to ciprofloxacin was high in thermophilic *Campylobacter* spp. from broilers in Turkey. It is well known that the irrational usage of antimicrobials in animal production is linked to the development of resistance in zoonotic bacteria and that the products of animal origin can be a source of transmission of resistant *Campylobacter* strains to humans (Owen and Leeton, 1999; Pearson et al., 2000). Fifty-nine per cent of human *Campylobacter* isolates were found to be resistant to quinolone in Turkey (Ongen *et al*., 2007).

Similar to other reports (Gaudreau and Gilbert, 1998; Rautelin *et al*., 1991), the 72 nalidixic acid-resistant *C. jejuni* isolates were also found to be resistant to ciprofloxacin and there was an absence of ciprofloxacin resistance in five nalidixic acid-resistant *C. jejuni* isolates in this study. That chicken isolates harbouring resistance to nalidixic acid remain susceptible to ciprofloxacin and that the Thr86-Ile substitution in *gryA* was the main substitution associated with high-level resistance to

quinolones (nalidixic acid) have been reported (Dionisi *et al*., 2004; Griggs *et al*., 2005; Kinana *et al*., 2007).

When evaluating the resistance of campylobacters, a high correlation between the agar disc diffusion method and the E-test for tetracycline (kappa = 0.91 for *C. jejuni* and kappa = 1.00 for *C. coli*) and quinolone/fluoroquinolones (kappa = 0.93 for *C. jejuni* and kappa = 0.97 for *C. coli*) was observed. This result is in agreement with previous reports, which evaluated the resistance of *Campylobacter* spp. to different antimicrobials by both tests (Rönner *et al*., 2004; Miflin *et al*., 2007). Therefore, this study also suggests that the disc diffusion method can be used as a reliable alternative for susceptibility testing of *Campylobacter* spp. to antimicrobial agents, particularly to tetracycline and quinolone/fluoroquinolones.

In conclusion, a decrease was found in the prevalence of thermophilic *Campylobacter* spp. in broilers in Turkey. The tetracycline/nalidixic acid/ciprofloxacin resistance pattern and tetracycline/nalidixic acid/ciprofloxacin/enrofloxacin resistance pattern were the predominant patterns in *C. jejuni* and *C. coli*, respectively. Another important result of this study is the absence of resistance to erythromycin as a macrolide, despite the high level of resistance to fluoroquinolones.

Acknowledgements

The authors wish to acknowledge to Munevver Atmaca and Ayse Uyar for technical assistance. This study was supported by grant 104T242 from The Scientific and Technological Research Council of Turkey, TUBITAK.

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