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The Prevalence of Multiple Antibiotic Resistance in *Campylobacter* spp. From Retail Poultry Meat**

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Summary

Macrolides and fluoroquinolones are regarded as drugs of choice for the treatment of human *Campylobacter* infections. The use of antimicrobials for this purpose as well as in food animal production has resulted in the resistance of *Campylobacter* spp. to selected antibiotics. Since poultry is one of the most important sources of human *Campylobacter* infections the use of antibiotics in animal production can shorten the effective therapeutic life of antibiotics for human use. During 2001–2003, over 220 strains of *C. jejuni* and *C. coli* were isolated from 60 poultry meat samples from the retail market in Slovenia and further characterized by phenotypic and molecular methods. In this study, 55 sample-representative strains were tested for susceptibility to eight different antibiotics (ampicillin, amoxycillin/clavulanic acid, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, pefloxacin and tetracycline). Phenotypic procedures (disc diffusion test, E-test) as well as molecular detection of mutations (mismatch amplification mutation assay (MAMA) polymerase chain reaction (PCR)) in case of ciprofloxacin resistance were used. When assuming the results about antibiotic resistance, only 38.2 % of strains tested were susceptible to all antibiotics tested. Regarding ciprofloxacin, 58.2 % of tested strains were found to be resistant (minimal inhibitory concentration, MIC>4 μg/mL). The occurrence of resistance was much higher in *C. coli* (75.9 %) than in *C. jejuni* (38.5 %) isolates. The resistance rates to pefloxacin, nalidixic acid, erythromycin and tetracycline were 58.2, 49.1, 14.5 and 12.7 %, respectively. Eleven percent of strains were resistant to erythromycin and ciprofloxacin and 12.7 % of strains were resistant to tetracycline and quinolones. The results show the need for monitoring the prevalence and antibiotic resistance of zoonotic bacteria such as *Campylobacter* as well as the multiresistance phenomenon of *Campylobacter* isolates from food in our country.

Key words: *Campylobacter*, poultry meat, antimicrobial resistance, multiple antibiotic resistance

Introduction

Infections with *Campylobacter* species have become the most common bacterial cause of human diarrhoea worldwide. The species *Campylobacter jejuni* and *C. coli* are associated with the majority of human infections (*1*,*2*). The incidence of campylobacteriosis continues to rise and it already exceeds the number of salmonellosis in many European countries (*3*,*4*). There is no simple explanation for this. In Slovenia, campylobacteriosis is the

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second most common foodborne infectious disease after salmonellosis. Between 1996 and 2000, the annual reported incidence of campylobacteriosis increased from 53 to 67.3 per 100 000 inhabitants (*5*).

A number of well studied as well as recently identified transmission vehicles and risk factors for *Campylobacter* spp. infection have been reported. The documented cause of the outbreak was ingestion of untreated surface waters and contaminated raw milk, although outbreaks are rare. Handling and consumption of contaminated undercooked poultry meat remains the most frequent source of human *Campylobacter* spp. infections (*6*). There were no official data available on the extent of the contamination of poultry meat with *Campylobacter* spp. in Slovenia. However, the results of our previous study indicated the high extent of retail poultry meat contamination with campylobacters (*7*).

Antibiotics are used in human and veterinary medicine for treatment and prevention of infections and as growth promotors in food animals (*8*). Their increased use has resulted in the increased incidence and levels of antibiotic resistance to many enteric bacteria and encouraged the persistence and transfer of antibiotic-resistance determinants in microbial genomes (*9–11*). Antibiotic resistance is an increasing problem in some pathogens and the capability of multidrug resistance is a concern. Some authorities regard food animals as the primary source of antibiotic resistance genes present in human food-borne pathogens, whereas the others as the major source of the problem regard imprudent use of antibiotics in humans (*12*). In case of zoonotic bacteria like campylobacters, the use of antimicrobials in animals may determine a drug-resistant bacterial population, which represents a potential threat to the consumer. When a pathogen has already acquired antibiotic resistance, there is no need for gene transfer or development of drug resistance for the pathogen to be difficult to control clinically (*11*).

Intestinal infections caused by *Campylobacter* spp. are usually self-limiting and therapy is not required, except in severe episodes of the disease, non-intestinal infections or in immunocompromised patients. Erythromycin and fluoroquinolone antibiotics, such as ciprofloxacin, are commonly used for treatment and prophylaxis (*13, 14*). Resistance to ciprofloxacin, which may result in treatment failure, has been rising in *Campylobacter* isolates from humans and animals after introduction of fluoroquinolones in veterinary medicine in the 1990s (*15*).

In Slovenia, the prevalence of thermotolerant campylobacters in human clinical isolates has been reported as well as their susceptibility to erythromycin and ciprofloxacin (*16*). However, the data about the prevalence of antibiotic resistance as well as about the multiresistance phenomenon in *Campylobacter* spp. from foods in Slovenia are not collected routinely at the national level. The present study was undertaken to evaluate the prevalence of resistant *Campylobacter* spp. in retail poultry meat in Slovenia. Antimicrobial susceptibility testing of poultry meat campylobacters was performed against eight different antibiotics used in veterinary and/or human medicine.

Materials and Methods

Sampling

Sixty samples of poultry meat (skin from legs and chicken liver) coming from eight different suppliers on Slovenian market were investigated between February 2001 and February 2003. Major integrated producing systems as well as small chicken meat producers were included. Fresh unwrapped meat or liver samples were purchased from butcher's shops and supermarkets, stored in sterile plastic bags, chilled and transported within 2 h to the laboratory for testing.

Isolation of Campylobacter spp.

Isolation of *Campylobacter* spp. was performed in accordance with the ISO 10272 guidelines (*17*). Samples were prepared aseptically by weighing 5 g into a sterile stomacher bag without filter, and then 45 mL of Preston enrichment broth (Oxoid) containing 5 % of horse blood (SR 048C, Oxoid, UK) was added and stomached for 120 s. The headspace was minimised for improved recovery. Thermophilic campylobacters were isolated after selective enrichment at 42 °C for 18 h. One loopful of enrichment broth was streaked on charcoal cefoperazone deoxycholate (CCDA, Oxoid, UK) agar plates and incubated in a microaerobic atmosphere (O_2 5 %, CO_2 10 %, N_2 85 %) in gas-tight containers at 42 °C for 3 days.

Species determination

Campylobacter jejuni and *C. coli* were identified by conventional tests and PCR molecular procedures. The absence of aerobic growth at 25 °C, of oxidase and catalase activity, and the absence of fermentation on glucose, sucrose and lactose were required specifities for thermotolerant campylobacters. A hydrolysis of hippurate was a biochemically distinguishing feature of *C. jejuni* against *C. coli* strains. Different PCR molecular procedures were used as described previously (*7*) to confirm phenotypic identification and omit mistakes because of unusual hippurate negative *C. jejuni* strains. Additionally, the identity of isolates was studied by PFGE typing using *Sma*I restriction endonuclease and CHEF mapper XA System (Bio-Rad Laboratories, USA) for 22 h at 200 V and 14 °C, for the separation of the fragments (*18,19*). The fingerprints obtained have been grouped into clusters, which have been found to be species specific (*20*).

Antimicrobial resistance testing

Testing was performed using disc diffusion method on Mueller-Hinton agar (211438, BD, USA) supplemented with horse blood (SR 048C, Oxoid, UK). The plates were incubated at (36±1) °C for 24–48 h in microaerobic atmosphere. The following antimicrobials were tested: ampicillin (10 μg), amoxycillin/clavulanic acid (20/10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), pefloxacin (5 μg), and tetracycline (30 μg). For testing and interpretation of the results we followed the general guidelines of NCCLS and Gaudreau and Gilbert (*21,22*). In case of ciprofloxacin and erythromycin resistance, MICs were determined by E-test. For the interpretation of the results, NARMS

interpretative criteria were used (Cipro-R, MIC>4 μg/mL and Erythro-R, MIC>8 μg/mL) (*23*).

The resistance to ciprofloxacin was confirmed also by mismatch amplification mutation assay (MAMA) polymerase chain reaction (PCR) detection of mutations in quinolone resistance determining region (QRDR) of the *gyrA* gene, by using primers, as described by Zirnstein *et al*. (*24,25*) and Zorman *et al.* (*26,27*).

Results and Discussion

Human enteric infections with *Campylobacter* spp. are common and responsible for considerable morbidity and occasional deaths. A large proportion of these infections have been attributed to chickens that are frequently contaminated with campylobacters (*28,29*).

Isolation of thermotolerant Campylobacter spp. from poultry meat

Sixty samples of retail poultry meat from eight Slovenian suppliers were examined for the presence of thermotolerant campylobacters. Most of the tested samples were found to be positive (54/60, 90 %). This contamination rate is even slightly higher than it was found in our previous work (*7*). Such results indicate a high extent of poultry meat contamination with campylobacters in Slovenia. Similar observations were also reported in studies from the USA, France, Great Britain and Italy (*30–33*), while reports from Scandinavian countries show much lower contamination (*34*). Thermotolerant campylobacters were found at least once in the samples from each supplier. Higher proportion of contaminated samples was found during summer and early autumn (100 %) compared to the samples from winter and early spring (67 %).

Phenotypic and genotypic identification of C. jejuni and C. coli

In total, over 220 thermotolerant *Campylobacter* strains were isolated following the ISO 10272 recommendations. In principle, taking one isolate from each sample, we chose 55 isolates for antimicrobial testing. Twenty-six of selected isolates (47 %) were identified as *C. jejuni* and 29 isolates (53 %) as *C. coli*. According to the published data, this is unusually high proportion of *C. coli* in poultry meat. In this calculation only 55 isolates were included, but similar proportion was also found (51 % of *C. jejuni* and 49 % of *C. coli*) in our previous study where 121 isolates from poultry meat purchased in two different geographical areas were included (*35*). In many reports, especially from the northern European countries, *C. jejuni* represents the most frequently isolated species (70–90 %) from poultry (*36–38*). The reason for this discrepancy could lie in the fact that different enrichment and isolation procedures were used in different studies. Preston enrichment broth, used also in our study, was reported to favour *C. coli* isolation (*39*). On the other hand, higher proportion of *C. coli* isolates from poultry meat in the southern European countries could be connected with the higher antimicrobial resistance observed in *C. coli* compared to *C. jejuni* (*33*). The results of identification have also shown that some samples of poultry meat were contaminated with *C. jejuni* and *C. coli* as well as with different strains of the same species.

Another interesting feature concerning species identification was a high proportion (35 %) of hippurate negative strains among *C. jejuni* isolates. Those isolates represent an unusual phenotype of *C. jejuni* and would be misidentified using only classical phenotypic identification.

Antimicrobial resistance

Most countries have already started national surveillance programs for monitoring the resistance of *Salmonella* while only a few countries appear to test *Campylobacter* regularly (*40*). Large differences in resistance profiles might be expected among countries, reflecting variations in the use of antibiotics (*41*). In Slovenia, no official data of the prevalence, antibiotic resistance and multiresistance of *Campylobacter* spp. from food are available.

The results of antimicrobial resistance testing of 55 isolates using disc diffusion method to screen for resistance against eight different antibiotics are shown in Table 1. In case of ciprofloxacin and erythromycin resistance, MICs were determined by E-test. The resistance to ciprofloxacin was confirmed also by MAMA-PCR detecting mutations in quinolone resistance determining region (QRDR) of the *gyrA* gene of *C. jejuni* and *C. coli.*

When assuming the results about antibiotic resistance, 21 out of 55 strains (38.2 %) of *Campylobacter* spp. were susceptible to all antibiotics tested.

In the overall resistance rates (except in the case of ampicillin, gentamicin and amoxycillin/clavulanic acid) a significant difference was found between *C. jejuni* and *C. coli* (Table 1). In our study, a higher portion of *C. jejuni* strains (61.5 %) was susceptible to all antibiotics tested, in comparison with *C. coli* strains (17.2 %). A similar situation was also reported previously (*29*). The resistance to one or more antibiotics tested was 61.8 % overall, with a significant difference between *C. jejuni* (38.5 %) and *C. coli* (82.8 %).

Ampicillin and other β-lactams are not recommended for the treatment of *Campylobacter* spp. infections due to the high amounts of β-lactamases produced by campylobacters (*41*) as well as because of the high incidence of intrinsic resistance to this family of drugs (*42*). Testing for β-lactamase production of strains was not done in our study. However, we found low incidence of resistance to ampicillin (1.8 %). Other studies report about much higher frequency of ampicillin-resistant *Campylobacter* spp. from different sources including poultry meat (*43–46*).

Tetracyclines were listed as an alternative treatment for campylobacter gastroenteritis in the past. They are widely used therapeutically and subtherapeutically as feed additives for livestock and poultry (*47*). In our study, the resistance to tetracycline was 12.7 % and it was found only in the strains resistant to quinolones. A significantly higher proportion of tetracycline-resistant isolates was found in *C. coli* (20.7 %) than in *C. jejuni* (3.8 %) isolates. Higher resistance rates of campylobacters to tetracycline have been reported by other researchers (*44,48*).

Table 1. Antimicrobial resistance of 55 *Campylobacter* isolates from retail poultry meat

Antibiotic	*C. jejuni*		*C. coli*		Total	
	n/N[c]	(%)	n/N[c]	(%)	n/N[c]	(%)
Ampicillin	1/26	(3.8)	0/29	(0.0)	1/55	(1.8)
Amoxycillin/clavulanic acid	0/26	(0.0)	0/29	(0.0)	0/55	(0.0)
Ciprofloxacin	10/26	(38.5)	22/29	(75.9)	32/55	(58.2)
Erythromycin	3/26	(11.5)	5/29	(17.2)	8/55	(14.5)
Gentamicin	0/26	(0.0)	0/29	(0.0)	0/55	(0.0)
Nalidixic acid	10/26	(38.5)	17/29	(58.6)	27/55	(49.1)
Pefloxacin	10/26	(38.5)	22/29	(75.9)	32/55	(58.2)
Tetracycline	1/26	(3.8)	6/29	(20.7)	7/55	(12.7)
Drug resistance[a]	10/26	(38.5)	24/29	(82.8)	34/55	(61.8)
Multiresistance[b]	3/26	(11.5)	3/29	(10.3)	6/55	(10.9)

[a] Resistance to one or more antimicrobial drugs
[b] Resistance to four or more antimicrobial drugs
[c] The number of resistant/the number of tested strains

No resistant strains to amoxycillin/clavulanic acid and gentamicin were found in our study. However, the resistance to erythromycin (MIC>8 μg/mL) among our tested isolates was 14.5 % (11.5 % in *C. jejuni* and 17.2 % in *C. coli*). The investigation of clinical isolates from England and Wales found erythromycin resistance in 1 % of *C. jejuni* and in 13 % of *C. coli* isolates (*49*). Some other countries report much higher frequency of erythromycin resistance in campylobacters. This is a concern since this drug is used therapeutically in human infections. In Spain, *C. coli* from pigs showed 81 % resistance to erythromycin, compared to 29 % from humans (*43*).

Concerning ciprofloxacin, studies of clinical isolates in England showed that resistance had risen from 3 % in 1991 to 12–19 % in 1997 (*50*). The presence of an efflux pump has been described as a possible mechanism for the development of fluoroquinolone resistance (*51*). However, a variety of mutations in the target topoisomerases, in particular the Thr-86→Ile mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, has been described more commonly (*52–54*). In our study, conventional testing by disc diffusion method was evaluated in comparison with the molecular procedure MAMA-PCR detecting point mutations on codon 86 of the *gyrA* gene. Since good correlation of the results was found, the comparison of both methods allowed identification of a few misinterpretations of the results by both approaches (*26,27*). Final determination of resistance showed that 58.2 % of our isolates were resistant to ciprofloxacin (MIC>4 μg/mL). The occurrence of resistance was much higher in *C. coli* (75.9 %) than in *C. jejuni* (38.5 %). These resistance rates are comparable with other European studies. A report from Belgium indicated that 42 % of *C. jejuni* and 62 % of *C. coli* isolates from broilers were resistant to ciprofloxacin (*48*) while in an Italian study, 53 % of *C. jejuni* isolates from chicken meat samples were resistant to quinolones (*33*). The extremely high prevalence of ciprofloxacin-resistant campylobacters from pigs and broilers (99 %) was reported from Spain (*43*).

The cross-resistance with ciprofloxacin was 100 and 84 % in case of pefloxacin and nalidixic acid, respectively. In overall, 58.2 and 49.1 % of tested strains were resistant to pefloxacin and nalidixic acid, respectively. Six out of 55 (10.9 %) strains (three *C. jejuni* and three *C. coli*) were resistant to ciprofloxacin and erythromycin (Cipro-R, MIC>4 μg/mL and Erythro-R, MIC>8 μg/mL).

Since 1990 there have been dramatic increases in the occurrence of multiple drug-resistant strains of zoonotic pathogens causing infections in humans in many developed countries (*48*). Multiresistance, defined as the resistance to four or more antibiotics tested, poses a threat to humans by limiting therapeutic choice of antibiotics. In this study, 10.9 % of strains showed the multiresistance profile (11.5 % for *C. jejuni* and 10.3 % for *C. coli*). This is a much higher rate than recently reported from Northern Ireland (*55*), where multiresistance for the isolates from broiler chickens was very low (0.8 % for *C. jejuni* and 0.0 % for *C. coli*). However, our results are comparable with the resistance studies of food animal isolates from Belgium (7.6 % of resistant strains of *C. jejuni* and 17.6 % of *C. coli*) (*49*) and of human isolates in England and Wales (11 % of resistant strains in *C. jejuni* and 20 % in *C. coli*) (*50*).

Conclusions

The results of our study demonstrated that the majority (90 %) of retail poultry meat samples from eight Slovenian suppliers harboured thermotolerant *Campylobacter* spp. Concerning antimicrobial resistance, 61.8 % of tested strains expressed resistance to at least one out of eight antimicrobials tested. High rate of resistance to ciprofloxacin was found among isolated *C. jejuni* (38.5 %) and *C. coli* (75.9 %) strains. Since the ingestion of the infected poultry meat may account for most of human campylobacteriosis cases, this fact could be important when ciprofloxacin is used for treatment of the patients. The resistance frequency to ampicillin, tetracycline, amoxycillin/clavulanic acid and gentamicin was comparable or lower than in the reports from most of the other European countries. However, multiple resistant strains were found among poultry meat isolates tested. These

results point out the need for monitoring the prevalence and antibiotic resistance of zoonotic bacteria in animals and humans, as well as in food, especially in the southern European countries, where microbial resistance rates seem to be higher than in other parts of Europe. These data could contribute to better understanding of the epidemiological links among the isolates from different sources and show the need for prudent use of antimicrobials in different fields of work, including the food production chain.

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Višestruka rezistentnost sojeva *Campylobacter* vrsta iz mesa peradi na antibiotike

Sažetak

Makrolidi i fluorokinoloni se najčešće koriste za liječenje ljudi od infekcija uzrokovanih sojevima *Campylobacter* vrsta. Korištenje antimikrobnih agensa pri liječenju ljudi te u

pripravi hrane za životinje uzrokovalo je povećanu antibiotičku rezistenciju sojeva *Campylobacter* vrsta. Budući da je piletina najčešći izvor infekcije ljudi sojem *Campylobacter*, primjena antibiotika u ishrani životinja može smanjiti njihov terapijski učinak na ljude. Od 2001. do 2003. godine izolirano je preko 220 sojeva vrsta *C. jejuni* i *C. coli* iz 60 uzoraka piletine iz trgovina u Sloveniji, a okarakterizirani su fenotipskim i molekularnim metodama. U radu je testirana osjetljivost 55 reprezentativnih sojeva iz dobivenih uzoraka prema 8 različitih antibiotika (ampicilin, amoksicilin/klavulanska kiselina, ciprofloksacin, eritromicin, gentamicin, nalidiksinska kiselina, pefloksacin i tetraciklin). Primijenjeni su fenotipski postupci (disk-difuzijski test, E-test), kao i molekularna detekcija mutacija (umnožavanje mutiranog alela pomoću specifičnog oligonukleotida – engl. mismatch amplification mutation assay – MAMA test) te polimerazna lančana reakcija (PCR) za utvrđivanje otpornosti ciprofloksacina. Sumirajući dobivene rezultate o antibiotičkoj otpornosti, samo 38,2 % ispitanih sojeva bilo je osjetljivo na sve korištene antibiotike. Na ciprofloksacin bilo je otporno 58,2 % od testiranih sojeva (MIC>4 μg/mL). Otpornost je bila veća u izolatima vrste *C. coli* (75,9 %) nego u vrsti *C. jejuni* (38,5 %). Otpornost na pefloksacin, nalidiksinsku kiselinu, eritromicin i tetraciklin iznosila je 58,2, 49,1 i 12,7 %. Na eritromicin i ciproflaksin bilo je otporno 11 % sojeva, a 12,7 % na tetraciklin i kinolone. Rezultati pokazuju da je potrebno provjeravati antibiotičku rezistentnost bakterija, kao što je rod *Campylobacter*, te pratiti višestruku rezistentnost izolata *Campylobacter* vrsta iz hrane.