Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter jejuni* and *C. coli* isolated from retail raw meat in Poland

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ABSTRACT: The study was conducted to investigate the presence of *Campylobacter* spp. in meat sold to consumers at a retail market in Poland. Antimicrobial resistance and the presence of putative virulence genes of the isolates were also examined. A total of 558 meat samples, including beef (n = 105), pork (n = 85), and poultry (n = 368) were collected over an almost three year study period. It was found that 321 samples, all of them originating from poultry, were contaminated with *Campylobacter* spp. Most of the obtained isolates were classified as *C. coli* (189 strains, 58.9%), whereas *C. jejuni* was identified in 132 (41.1%) samples. All *Campylobacter* strains were susceptible to gentamicin and all but one *C. coli* isolate to erythromycin. On the other hand, the highest level of resistance among *Campylobacter* tested was to ciprofloxacin (91% for *C. jejuni* and 86.1% for *C. coli*) and nalidixic acid (89.3% for *C. jejuni* and 85% for *C. coli*). Furthermore, resistance to two or more classes of antibiotics was found in the majority (60.9%) of *Campylobacter* spp. and among them one *C. coli* strain showed resistance to four different classes of antimicrobials. Identification of virulence genes in the isolated *Campylobacter* showed that all of them had the *flaA* and *cadF* genes. The *iam* marker was found more often in *C. coli* strains (88.8%) compared to *C. jejuni* isolates (53.8%). On the other hand, the *virB11* gene was identified only in 4.2% of *C. coli* and in 6.1% of *C. jejuni* strains, respectively. Furthermore, the prevalence of the *cdtA*, *cdtB*, and *cdtC* genes among *C. jejuni* strains was 97.7%, 93.2%, 96.2%, respectively, and was significantly higher than for *C. coli* regarding the *cdtC* (66.7%) gene. The obtained results showed that the presence of *Campylobacter* in retail meat may represent a threat to public health.

Keywords: *Campylobacter* spp.; retail meat; antimicrobial resistance; virulence factors

Campylobacter, which includes *C. jejuni* and *C. coli*, is the main pathogen causing foodborne diseases worldwide (Scallan et al. 2011; Anonymous 2012a). According to European Food Safety Authority (EFSA) reports, campylobacteriosis is still the most commonly reported zoonosis in the European Union (EU) with 212 064 confirmed cases in 2010. Additionally, from 2006 a significant upward trend in the number of infection cases has been observed. *Campylobacter* is widely distributed in poultry; however, cattle, pigs, sheep, and pet animals may also be a source of these microorganisms. *Campylobacter* is most often detected in fresh broiler meat and in the EU the prevalence of these bacteria in broiler carcasses identified at the retail level varied from 3.1% to 58.8%, depending on the Member State (MS) (Anonymous 2010, 2012a).

Human *Campylobacter* infection may be due to either consumption of undercooked meat or cross-contamination of ready-to-eat food during preparation or storage. Campylobacteriosis is often self-limiting and does not require antimicrobial treatment. However, in some cases such as septicaemia or other invasive forms of the disease, characterised by severe and prolonged enteritis as well as in immunocompromised or very young patients, antibacterial therapy may be needed. Macrolides (erythromycin) and quinolones, including fluoroquinolones (ciprofloxacin, nalidixic acid), are usually used in the treatment of *Campylobacter* infections. In recent years increasing numbers of resistant *Campylobacter* isolates, especially to quinolones, have been observed (Anonymous 2012a).

Although *Campylobacter* is a leading cause of foodborne illnesses, little is known about the mechanism of gastroenteritis induction in humans. The lack of understanding concerning the pathogenic mechanism has limited the prevention of human

infection. However, several studies showed that certain bacterial factors are essential for the pathogenesis of campylobacteriosis, including the motility and adherence of bacteria to intestinal mucosa, capability to invade enterocytes as well as toxin production (Datta et al. 2003; Dasti et al. 2010). Moreover, some potential genetic markers of bacterial virulence have been identified such as *flaA* and *cadF* involved in adhesion and colonisation, *virB11* and *iam* associated with invasiveness, as well as the *cdtA*, *cdtB* and *cdtC* toxin genes encoding *Campylobacter* cytotoxins (Young et al. 2007; Dasti et al. 2010; Rapabelli et al. 2010).

The aim of the study was to determine the prevalence of *Campylobacter* in retail meat available in Poland. Additionally, the isolated strains were characterised for antimicrobial resistance and the presence of putative virulence markers.

MATERIAL AND METHODS

Meat samples

A total of 558 retail meat samples were purchased between April 2009 and December 2011 from local supermarkets in the eastern part of Poland. Over the course of the study 105 beef, 85 pork, and 368 poultry meat samples were analysed. The chicken samples included different parts of the carcasses such as wings (n = 67), legs (n = 175), corpuses (n = 49), and breast fillets (n = 77).

Isolation and enumeration of *Campylobacter*

The detection and enumeration of *Campylobacter* isolates were performed according to the ISO 10272-1:2006 and ISO/TS 10272-2:2006 standards, respectively, using microaerophilic conditions generated by the Campy Gen gas-generating kit (Oxoid, UK). The bacterial isolates were confirmed as *C. jejuni* and *C. coli* using a PCR method as described previously (Wieczorek and Osek 2005). The strains were stored at –80 °C until further analysis.

Identification of putative *Campylobacter* virulence genes

The presence of seven *Campylobacter* virulence genes (*iam*, *flaA*, *cadF*, *virB11*, *cdtA*, *cdtB*, *cdtC*) was tested using PCR with primers (Symbios, Poland) and amplification conditions as described in Table 1. The generated PCR amplicons were stained with ethidium bromide, visualised in 2% agarose gels (Sigma, USA) in Tris-Acetate-EDTA and photographed using the Gel Doc 2000 documentation system (Bio-Rad, USA).

Table 1. PCR primers and amplification conditions used to identify *Campylobacter* virulence genes

Target gene	Primer name	Oligonucleotide sequence (5'→3')	Amplicon size (bp)	Annealining temperature (°C)	References
iam	IAMF IAMR	GCGCAAATATTATCACCC TTCACGACTACTACTATGCGG	518	55	Korsak et al. (2004)
virB11	VirBF VirBR	GAACAGGAAGTGGAAAAACTAGC TTCCGCATTGGGCTATATG	708	55	Bacon et al. (2000)
flaA	fla AF flaAR	GGATTTCGTATTAACACAAATGGTGC CTGTAGTAATCTTAAACATTTTG	1700	48	Wieczorek and Osek (2008)
cadF	F2B R1B	TGGAGGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC	400	45	Konkel et al. (1997)
cdtA	GNW IVH	GGAAATTGGATTTGGGGCTATACT ATCAACAAGGATAATGGACAAT	165	55	Rapabelli et al. (2010)
cdtB	VAT2 WMI-R	GTTAAAATCCCTGCTATCAACCA GTTGGCACTTGGAATTTGCAAGGC	495	57	Rapabelli et al. (2010)
cdtC	WMI-F LPF-X	TGGATGATAGCAGGGGATTTTAAC TTGCACATAACCAAAAGGAAG	555	55	Rapabelli et al. (2010)

Table 2. Antimicrobials and cut-off values used for MIC determination of the tested *Campylobacter*

Antimicrobial groups	Antimicrobial	MIC (mg/l)		Number/(%) of resistant strains		
		C. jejuni	*C. coli*	*C. jejuni* (*n* = 122)	*C. coli* (*n* = 180)	total (*n* = 302)
Quinolones and Fluoroquinolones	ciprofloxacin	1	1	111/(91.0)	155/(86.1)	266/(88.1)
	nalidixic acid	16	32	109/(89.3)	153/(85.0)	262/(86.8)
Macrolides	erythromycin	4	16	0	1/(0.8)	1/(0.3)
Tetracyclines	tetracycline	2	2	60/(49.1)	114/(63.3)	174/(57.6)
Aminoglycosides	gentamycin	1	2	0	0	0
	streptomycin	2	4	13/(10.7)	56/(31.1)	69/(22.8)

Determination of antimicrobial resistance

Campylobacter isolates were sub-cultured twice on Columbia agar supplemented with 5% sheep blood (Oxoid) and incubated at 41.5 °C for 44 ± 4 h in microaerophilic conditions. After incubation, a suspension equivalent to 0.5 McFarland standard was prepared and transferred to Mueller-Hinton broth supplemented with 5% of sheep blood (Trek, UK) and 100 µl was used to inoculate antibiotic plates (Sensitire *Campylobacter* Plate-EUCAMP). The plates were incubated in microaerophilic conditions for 48 h at 37 °C and the minimal inhibitory concentration (MIC) records were then read using the Vision system (Trek, UK). The antimicrobials and cut off values used for the interpretation of the MIC results were in accordance with EUCAST (www.eucast.org) and the EU Community Reference Laboratory for Antimicrobial Resistance (Table 2).

RESULTS

In total, 558 retail meat samples were analysed for the presence and number of *Campylobacter* spp. The bacteria were only identified in poultry meat whereas porcine and bovine samples were all negative. It was found that 321 out of 368 (87.2%) parts of chicken carcasses were contaminated with *Campylobacter*. PCR identification revealed that *C. coli* was detected in 189 samples (58.9%), whereas *C. jejuni* was identified in the remaining 132 (41.1%) positive samples. Regarding quantitative results, *Campylobacter* was found at an enumerable level (> 10^2 cfu/g) in 65 out of 321 (20.2%) samples (Table 3).

The results of the identification of virulence markers among *Campylobacter* tested in the study are presented in Figure 1. All isolates, irrespective of the bacterial genus, were positive for the *cadF* and *flaA* genes. Furthermore, the *cdt* toxin genes were identified in most of the isolates tested. A higher rate of *iam* marker occurrence was found in *C. coli* isolates (88.8%) as compared with *C. jejuni* (53.8%). On the other hand, the *virB11* gene was identified only in 4.2% *C. coli* and in 6.1% *C. jejuni* strains, respectively (Figure 1).

The antimicrobial resistance of *Campylobacter* was determined in 302 out of the 321 obtained isolates since the remaining 19 strains did not grow on the antimicrobial plates. The results are presented in Tables 2 and 4. All isolates were susceptible to gentamicin and erythromycin except for one *C. coli* strain. The highest resistance rate was found to

Table 3. Prevalence and number of *Campylobacter* isolated from raw chicken samples

Sample type	Number of samples tested/positive (%) for *Campylobacter*	Number/(%) of samples positive for		Contamination level (cfu/g) – number/(%) of samples			
		C. coli	*C. jejuni*	< 10^2	10^2–10^3	10^3–10^4	> 10^4
Wings	67/58 (86.6)	39/(58.2)	19/(28.3)	48/(82.7)	7/(12.1)	3/(5.2)	
Legs	175/156 (89.1)	85/(48.5)	71/(40.6)	114/(73.1)	19/(12.1)	21/(13.5)	2/(1.3)
Corpuses	49/43 (87.7)	25/(51)	18/(36.7)	33/(76.7)	3/ (7)	6/(14)	1/(2.3)
Breast filets	77/64 (83.1)	40/(52)	24/(31.2)	61/(95.3)	1/(1.6)	2/(3.1)	
Total	368/321 (87.2)	189/(51.4)	132/(35.9)	256/(79.8)	30/(9.3)	32/(10)	3/(0.9)

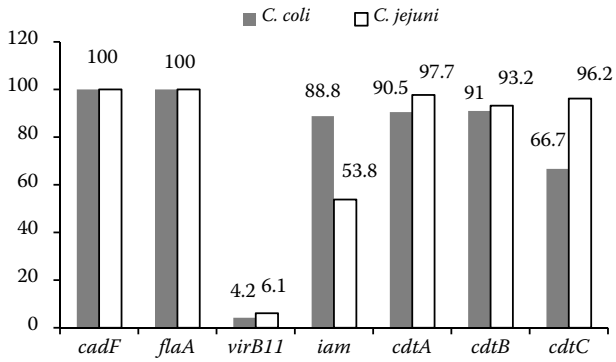

Figure 1. The presence (%) of virulence genes in *Campylobacter* species isolated from poultry

quinolones, where 88.1% and 86.8% of the isolates were resistant to ciprofloxacin and nalidixic acid, respectively (Table 2). There was no significant difference in the susceptibility to these drugs between *C. coli* and *C. jejuni* isolates. The majority of the isolates were also resistant to tetracycline, although the percentage of *C. coli* (63.3%) was higher than that of *C. jejuni* (49.2%) (Table 2). Several strains were also resistant to streptomycin and more *C. coli* (31.1%) than *C. jejuni* (10.6%) isolates displayed this antimicrobial property. On the other hand, 36 *Campylobacter* strains were susceptible to all seven antimicrobials used in the study. Resistance to two or more classes of antibiotics was found in 184 (60.9%) of *Campylobacter* spp. and among them one *C. coli* strain revealed resistance to four antimicrobial groups (Table 4). The most common resistance pattern observed among *C. jejuni* and *C. coli* was Cip Nal Tet, where 114 (37.7%) of the isolates were identified (Table 4).

DISCUSSION

In the present study 321 out of a total of 558 examined samples were *Campylobacter*-positive; it should be underlined that all of them were of poultry origin (321 out of 368 poultry meat samples tested; 87.2%). Among 105 beef and 85 pork samples tested none were positive. Other EU MSs also reported a low proportion of *Campylobacter*-positive fresh pig or beef meat samples at the retail level, or even no isolation at all (Whyte et al. 2004; Zhao et al. 2010; Anonymous 2012a). Comparing the frequency of occurrence of *Campylobacter* in different parts of chicken carcasses it was found that the legs were the most often contaminated with *Campylobacter* (89.1%), followed by corpuses (87.7%), wings (86.6%) and breast fillets (83.1%), although the differences in the prevalence of *Campylobacter* in various chicken parts were not significant (Table 3). In 2008, an extensive survey on the prevalence of *Campylobacter* spp. in broiler carcasses from slaughterhouses in the European Union was carried out and showed that 75.8% of samples were contaminated with these bacteria. In Poland, 83% positive carcasses were found whereas other MSs reported different levels of contamination – from 4.9% in Estonia to 100% in Luxembourg (Anonymous 2010). Taking into account the data from the present study with poultry meat purchased at the retail level, it can be seen that the percentage of *Campylobacter*-positive samples was higher than the average prevalence in the EU. However, the samples were collected at different production stages. Furthermore, other studies con-

Table 4. Antimicrobial resistance phenotype patterns among the tested *Campylobacter*

Antimicrobial resistance phenotype	Number of different antibiotic classes	Number/(%) of resistant isolates		
		C. coli (n = 180)	*C. jejuni* (n = 122)	total (n = 302)
Sentitive for all	0	25/(13.8)	11/(9.0)	36/(11.9)
Cip	1	0	2/(1.6)	2/(0.6)
Cip Nal	1	34/(18.8)	46/(37.7)	80/(26.5)
Cip Tet	2	1/(0.5)	0	1/(0.3)
Cip Nal Str	2	7/(3.8)	3/(2.5)	10/(3.3)
Cip Nal Tet	2	64/(35.5)	50/(41)	114/(37.7)
Cip Str Tet	3	1/(0.5)	2/(1.6)	3/(0.9)
Cip Nal Str Tet	3	47/(26.1)	8/(6.5)	55/(18.2)
Cip Nal Str Tet Ery	4	1/(0.5)	0	1/(0.3)

Cip = ciprofloxacin, Nal = nalidixic acid, Tet = tetracyclin, Str = streptomycin, Ery = erythromycin

cerning the prevalence of *Campylobacter* at the retail level showed a lower proportion of positive samples than obtained in the present investigations (Prencipe et al. 2007; Lynch et al. 2011). On the other hand, there are some reports showing a higher rate contamination in similar samples, even over 90% (Rozynek et al. 2008; Tang et al. 2009; Mackiw et al. 2012).

Additionally, the number of *Campylobacter* in the tested samples was estimated and it was noted that 20.2% of them were contaminated with bacteria at an enumerable level, i.e. over 100 cfu/g (Table 3). Among them, less than 1% of samples harboured high numbers of microorganisms, i.e. over 10^4 cfu/g. Most of the chicken meat parts tested in the present study contained a relatively low number of *Campylobacter* that could be enumerated with the method used. In the above mentioned EFSA report, 46.6% poultry samples revealed a number of *Campylobacter* below 10 cfu/g; however, in 5.8% of samples the number of *Campylobacter* was over 10^4 cfu/g (Anonymous 2010).

Analysis of *Campylobacter* species identified in the present study revealed that most of them were *C. coli* (58.9%) while *C. jejuni* was detected in the remaining 41.1% poultry meat samples. These findings are in contrast to data obtained by other authors who in similar samples detected mostly *C. jejuni* (Sallam 2007; Rozynek et al. 2008; Bardon et al. 2011; Anonymous 2012a). However, there are also some reports were *C. coli* was more predominant than *C. jejuni* in poultry meat (Kurincic et al. 2005; Lynch et al. 2011; Mackiw et al. 2012).

In recent years several studies have confirmed the increased number of *Campylobacter* isolates resistant to macrolides and fluoroquinolones. These antibiotics are considered as the drugs of choice for the treatment of human gastroenteritis infections, so the increased resistance of such strains poses a public health problem (Alfredson and Korolic 2007; Anonymous 2012b). In the current study the susceptibility of *Campylobacter* isolates to seven antimicrobials was determined. The highest resistance rate was observed to quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin). As described in the EFSA report (Anonymous 2012b) resistance to these groups of antimicrobials is predominant among *Campylobacter* isolates of poultry meat origin in many EU MSs. However, other studies, especially those conducted in the Nordic countries, showed low resistance rates among these microorganisms isolated from chicken meat (Frediani-Wolf and Stephan 2003; Andersen et al. 2006; Bardon et al. 2011). Our data on the susceptibility of *Campylobacter* isolates to ciprofloxacin are similar to the results obtained in some other European countries, e.g. Slovenia and Austria, where 78% of *C. jejuni* and 79% of *C. coli* strains of poultry meat origin were found to be resistant, respectively (Anonymous 2012b).

Since erythromycin is the drug of choice for the treatment of *Campylobacter* infections the prevalence of resistance to this antimicrobial, especially among strains isolated from food, should be a cause for special concern. Previous studies on the susceptibility of *Campylobacter* to macrolides showed that the percentage of resistant isolates was at a low level and did not exceed 1% (Andersen et al. 2006; Rozynek et al. 2008; Wozniak and Wieliczko 2011). The findings of the present investigation are consistent with those results since only one *C. coli* out of 302 isolates tested was resistant to erythromycin (Table 2). On the other hand, some EU MSs reported a relatively high level of resistance to erythromycin, e.g., 4% and 18% in Belgium for *C. jejuni* and *C. coli*, respectively or 39% of the *Campylobacter* isolates in the Netherlands (Anonymous 2012b). Furthermore, a relatively low level of resistance of the strains tested to streptomycin (22.8%) obtained during our study is similar to other data where the percentage of such strains was higher among *C. coli* than *C. jejuni* (McGill et al. 2006; Bardon et al. 2011). In the present investigation none of the *Campylobacter* strains was resistant to another antimicrobial from the aminoglycoside group – gentamicin. It was also observed that resistance to tetracyclines (63.3% for *C. coli* and 49.2% for *C. jejuni*) was on a similar level to that recorded by other authors (Zhao et al. 2010; Wozniak and Wieliczko 2011; Anonymous 2012b). However, the data presented by Andersen et al. (2006) and Rozynek et al. (2008) suggested an increasing tendency in the incidence of resistant strains to tetracycline isolated from chicken meat.

In the present study the vast majority of the *Campylobacter* strains (88.1%) were resistant to one or more antibiotics. Moreover, most of the isolates tested (60.9%) revealed resistance to two or more different classes of antimicrobials and this percentage was higher than that reported by other authors (Andersen et al. 2006; Sallam 2007; Rozynek et al. 2008). It should also be underlined that one *C. coli* strain resistant to four groups of antimicrobials including fluoroquinolones and macrolides, was identified.

The *flaA* (engaged in motility and colonization), *cadF* (encoding fibronectin-binding outer membrane protein), and *cdtABC* markers (responsible for toxin producing) were detected in a high percentage of the isolates tested (Figure 1). These results are similar to data previously reported by other authors (Datta et al. 2003; Rozynek et al. 2005; Krutkiewicz and Klimuszko 2010; Rapabelli et al. 2010). The invasion-associated marker (*iam*) of *Campylobacter* was another virulence marker detected in this study. More than 88% of *C. coli* and nearly 54% of *C. jejuni* isolates harboured this gene. Such differences in the prevalence of the *iam* factor were also found by other authors (Korsak et al. 2004; Rozynek et al. 2005). Furthermore, these studies suggest that this virulence marker is not only essential for the colonisation of the chicken gut but is also responsible for the induction of diarrhoea in humans (Korsak et al. 2004; Rozynek et al. 2005).

It was also observed that the *virB11* gene, localised on the pVir plasmid was found only in small number of the *C. coli* (4.2%) and *C. jejuni* (6.1%) strains tested. This is in contrast to the data obtained by other authors (Datta et al. 2003; Rizal et al. 2010). However, the role of this gene and its product in the pathogenesis of campylobacteriosis is still not clear (Tracz et al. 2005; Louwen et al. 2006; Nielsen et al. 2010).

In conclusion, this survey revealed that raw poultry meat available for consumers in Poland was often contaminated with *Campylobacter*. Furthermore, a high rate of resistance to quinolones and resistance to more than one class of antibiotics among *Campylobacter* isolates was found. Additionally, several strains were positive for the *flaA*, *cadF*, and *cdt* putative virulence marker genes. All these findings suggest that the consumption of undercooked meat or food cross-contaminated with *Campylobacter* may pose a serious threat to consumer health.

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Received: 2012–06–22
Accepted after corrections: 2012–07–07

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