International Journal of Food Microbiology 210 (2015) 24–32

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Comparative analysis of antimicrobial resistance and genetic diversity of *Campylobacter* from broilers slaughtered in Poland

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ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 12 May 2015
Accepted 9 June 2015
Available online 11 June 2015

Keywords:
Campylobacter
Poultry
Antimicrobial resistance
PFGE
Virulence genes
Composite analysis

ABSTRACT

In the current study, the relationship of *Campylobacter jejuni* and *Campylobacter coli* strains isolated at slaughter was investigated using comparative analysis of antimicrobial resistance (AMR), virulence gene (VG) and PFGE profiling. A total of 254 *Campylobacter* isolates from poultry caeca and corresponding carcasses, including 139 *C. jejuni* and 115 *C. coli* strains were tested. The most prevalent resistance profiles observed in *C. jejuni* were ciprofloxacin, nalidixic acid and tetracycline (46 out of 139, 33.1% isolates) as well as ciprofloxacin, nalidixic acid, tetracycline and streptomycin among *C. coli* strains (34 out of 115, 29.6%). Multi-resistance was found more frequently among *C. coli* than *C. jejuni* ($P < 0.05$). The presence of 11 virulence genes exhibited 19 different VG profiles in *Campylobacter* isolates tested. All *Campylobacter* strains were classified into 154 different PFGE types. Among them, 56 profiles (28 *C. jejuni* and 28 *C. coli*) were common for at least two isolates including 9 clusters covering from 4 to 9 strains. *Campylobacter* composite types generated by a combination of 154 PFGE types, 10 AMR profiles and 19 VG patterns divided 178 distinct types with 95% similarity. The majority of the composite profiles (76 for *C. jejuni* and 58 for *C. coli*; 75.3% in total) included only one bacterial isolate. Furthermore, 11 pairs of *C. jejuni* and 12 pairs of *C. coli* from caeca and the corresponding carcasses isolated from the same places possessed the identical PFGE, AMR and VG patterns.

This study demonstrated that *C. jejuni* and *C. coli* isolated from poultry in Poland showed to have a high genetic diversity and a weak clonal population structure. However, the composite analysis revealed a strong evidence for cross-contamination of chicken carcasses during the slaughter process. Additionally, our results confirm that *Campylobacter* may easily contaminate poultry carcasses at slaughter process and spread around country. More than half of *Campylobacter* strains tested (50.4%) were resistant to at least two classes of antimicrobials, i.e. quinolones and tetracyclines, which may cause a public health risk.

1. Introduction

Consumption of undercooked poultry meat or other products cross-contaminated with *Campylobacter* during processing is recognized as one of the main sources of campylobacteriosis in humans (EFSA and ECDC, 2015). These microorganisms are a part of normal gastrointestinal microflora in many animals, especially birds (Epps et al., 2013). Chickens can be infected with the bacteria at a very high level without any clinical symptoms and *Campylobacter* is frequently isolated from the caecal microflora (Agunos et al., 2014; Lindmark et al., 2009). Intestinal content is therefore suspected to be the main source of broiler carcass contamination at slaughter (Kudirkiene et al., 2011). Number of studies revealed that chicken meat has a significant role in development of campylobacteriosis in humans (Abay et al., 2014; Kittl et al., 2013; Lindmark et al., 2009). The European Union (EU) Member States have recently monitored incidence of *Campylobacter* in poultry and poultry meat, and the results indicated that the level of bacteria prevalence varied widely from 0 to 80.6% or 83.6%, regarding fresh broiler meat and broilers, respectively (EFSA and ECDC, 2015).

Chicken meat and its products are commonly consumed in Poland, but campylobaceriosis is rarely reported compared to other European countries. In 2013, according to the EFSA report 1.4 confirmed cases per 100,000 inhabitants were noted in Poland compared to the average EU reported level of 64.8 (EFSA and ECDC, 2015). The disease is usually self-limiting; however, in severe cases antimicrobial therapy is required. Two groups of antibiotics are most commonly used in treatment of campylobacteriosis: macrolides and fluoroquinolones. That is why the increased resistance of *Campylobacter* of human origin to these antimicrobials observed worldwide is cause for interest from a public health perspective (Ge et al., 2013; Luangtongkum et al., 2009). Additionally, knowledge about *Campylobacter* antimicrobial resistance at different levels of poultry production is important for development of effective control strategies. Several studies have been conducted to investigate the prevalence and *Campylobacter* antimicrobial susceptibility patterns and most of them reported the emergence of resistant strains, especially to quinolones (Agunos et al., 2014; Cardinale et al., 2006; Fraqueza et al.,

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.06.006
0168-1605/

2014; Messad et al., 2014; Nobile et al., 2013; Wimalarathna et al., 2013).

Despite the importance of *Campylobacter* as a foodborne pathogen, the exact mechanisms by which *Campylobacter jejuni* or *Campylobacter coli* cause infection are unknown. However, some factors that attribute to *Campylobacter* pathogenicity in humans, i.e. motility (determined by the *flaA* and *flhA* genes), host cell adherence and cell invasion (encoded by the *cadF*, *docA*, *ciaB*, *iam*, *wlaN*, and *virB11* markers) as well as induction of host cell death by toxin production (*cdtA*, *cdtB*, *cdtC* markers) are essential to cause the illness (Epps et al., 2013).

For tracking of *Campylobacter* infection and genotypic characterization of phenotypically similar isolates different molecular typing methods have been developed. Among them, macrorestriction analysis with pulsed-field gel electrophoresis (PFGE) is used as a gold standard due to its high discrimination potential. PFGE is commonly used to differentiate isolates from different reservoirs such as humans, animals, environmental samples and transmission of these bacteria (Ahmed et al., 2012; Taboada et al., 2013). This molecular analysis has shown that *C. jejuni* and *C. coli* are genetically diverse and investigations of several *Campylobacter* collections usually demonstrated high strain variability regardless of genotyping methods used (Abay et al., 2014; Damjanova et al., 2011; Di Giannatale et al., 2014; Lazou et al., 2014; Ma et al., 2014; Magnusson et al., 2011; Melero et al., 2012; Thakur et al., 2013).

The objective of this study was to investigate a clonal relationship among poultry *Campylobacter* isolates in Poland. For this purpose, antimicrobial-resistance, virulence gene patterns and PFGE profiles of *C. jejuni* and *C. coli* recovered from caeca and the corresponding carcasses were examined.

2. Materials and methods

2.1. Campylobacter isolates

Altogether, 254 *Campylobacter* isolates from broiler's caeca (101 strains) and carcasses (153 isolates) collected in Poland over the period from June 2011 to May 2012 were used in this study. The territory of Poland was divided to 8 regions with two voivodeships (Polish administrative unit) in each. The samples from each region were collected every month and their number depended on the number of holdings located in each voivodeship. The number of *Campylobacter* isolates tested from each region is shown in Table 1. During each visit, intact caeca from 10 birds of one flock were taken immediately after evisceration and one whole carcass after chilling was collected and transported to the laboratory within 24 h at 2–8 °C. In the next step, the caecal samples were pooled, streaked directly on Karmali agar (*Campylobacter* Agar Base + *Campylobacter* Selective Supplement; Oxoid, UK) and incubated at 41.5 ± 1 °C for at least 48 +/− 2 h in a microaerobic atmosphere generated using CampyGen kit (Oxoid). Subsequently, typical *Campylobacter* colonies were selected for further investigation with ISO 10272-1 method. *Campylobacter* were isolated from carcasses using the ISO 10272-1 standard. From each positive caecal and carcass samples one bacterial isolates were identified and speciated with PCR as described previously (Wang et al., 2002; Wieczorek and Osek, 2005).

2.2. Antimicrobial susceptibility

The set of six antimicrobials belonging to five different classes, i.e. aminoglicosides (gentamicin, GEN and streptomycin, STR), macrolides (erythromycin, ERY) quinolones and fluoroquinolones (nalidixic acid, NAL and ciprofloxacin, CIP), and tetracyclines (tetracycline, TET) was used for determination of AMR profiles of the isolated *C. jejuni* and *C. coli*. The microbroth dilution method to establish MIC (minimal inhibitory concentration) values was used as described before by Wieczorek et al. (2013). Briefly, the isolates were subcultured twice on Columbia agar (Oxoid) and the bacteria were suspended in Mueller-Hinton Broth supplemented with 2–2.5% horse blood (Trek Diagnostic, UK) and transferred to the commercially prepared Eucamp microtitre plates (Trek Diagnostic) with the following dehydrated antimicrobials: GEN (0.12–16 mg/L), STR (1–16 mg/L), ERY (0.5–32 mg/L), CIP (0.06–4 mg/L), NAL (2–64 mg/L), and TET (0.25–16 mg/L). The plates were incubated for 44 ± 4 h at 37 °C in microaerophilic atmosphere.

2.3. Detection of virulence genes

PCR was used for identification of 11 *Campylobacter* virulence genes (VGs): *flaA*, *flhA*, *cadF*, *docA*, *cdtA*, *cdtB*, *cdtC*, *ciaB*, *iam*, *wlaN*, and *virB11* as previously described by Wieczorek et al. (2013).

2.4. Pulsed-filed gel electrophoresis (PFGE)

The procedure with *Sma*I restriction enzyme (Fermentas, EU) as described by Ribot et al. (2001) was used to perform the PFGE analysis. The gels were stained with ethidium bromide and photographed under ultraviolet light with the Gel Doc 2000 system (Bio-Rad, USA).

2.5. Reference strains

The following reference strains were included in the study: *C. jejuni* ATCC 33560 (the quality control strain in antimicrobial susceptibility testing and positive control in PCR analyses), *C. coli* ATCC 43478 (positive control in PCR), and *Salmonella* Braenderup H9812 ATCC BAA-664 (molecular weight size standard in PFGE).

2.6. Analysis of molecular patterns

PFGE images were analyzed with Bionumerics software version 6.6 (Applied Maths, Belgium). Band matching and cluster analysis were accomplished using Dice correlation co-efficient for similarity and the unweighted-pair group method with arithmetic means (UPGMA). Optimization parameters of 1% and DNA bands position tolerance of 2% were

Table 1
Comparison of antimicrobial resistance, virulence gene patterns and PFGE profiles of *Campylobacter* isolated from different regions in Poland.

Region code	*C. jejuni*						*C. coli*					
	Number of isolates		Pattern number		Number of profiles		Number of isolates		Pattern number		Number of profiles	
	Caeca (n = 55)	Carcasses (n = 84)	AMR	VG	PFGE	Composite analysis	Caeca (n = 46)	Carcasses (n = 69)	AMR	VG	PFGE	Composite analysis
KI	7	10	1,3,4,6,8	3,11,14,16	12	14	3	9	1,3,4,6,8	4,5,6,7,15,18	10	10
KR	4	12	1,4,6,8	8,11,14,15,16,18	10	13	11	11	1,3,4,6,7,8,10	1,5,9,15,19	13	19
L	8	7	1,4,6	8,11,14,15,16,17	11	13	3	6	1,4,6,8	5,15	8	8
O	3	7	1,4,6,8	10,11,14,15,16	9	8	6	8	1,4,6,7,8	5,9,15	11	12
P	12	21	1,4,6,8	8,10,13,14,15,16	24	26	8	11	1,3,4,6,7,8,9	1,2,5,12,15	14	16
S	7	15	1,4,6,8	3,14,15,17	17	18	7	11	1,4,6,7,8	5,6,12,15	13	15
WA	6	5	2,4,6,8	14,15,16	9	9	4	5	1,4,8	5,12,15	9	6
WR	8	7	1,3,4,6,7,8	14,16,17	13	13	4	8	1,2,4,5,6,7,8	5,12	9	9