The prevalence of four virulence genes in strains of *Campylobacter jejuni* isolated from broilers in Serbia

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ABSTRACT

Campylobacter jejuni is a major cause of human diarrheal disease. The objective of this research study was to determine the prevalence of different virulence genes in isolates recovered from broiler meat at slaughterhouses in Serbia. Out of 115 *Campylobacter* spp. samples recovered, a total of 35 isolates were identified as *C. jejuni* on the basis of morphological, biochemical-based detection, multiplex PCR, and sequencing of the highly conserved region of the *dnaJ* gene encoding the DnaJ Hsp40 family protein. The isolates were screened for the presence of four pathogenic genes, namely *flaA*, *cadF*, *cdtB*, and *cgtB*, which are responsible for the expression of adherence, colonization, cytotoxin production in *C. jejuni,* and the onset of Guillain-Barre syndrome. The isolates showed a wide variation in the presence of these genes. All the isolates were positive for *flaA*. Furthermore, a high genetic heterogeneity in the *C. jejuni* population was found in this study, showing a pattern partially different from other reported virulence genes. Of the *C. jejuni* studied, 94.3%, 97.1% and 5.7% were positive for *cadF*, *cdtB* and *cgtB*, respectively. This study provides initial data on the prevalence and distribution of the *flaA*, *cadF*, *cdtB*, and *cgtB* genes in *C. jejuni* isolated from broiler meat in Serbia.

Key words: slaughterhouse; *C. jejuni*; virulence genes

Introduction

Thermophilic *Campylobacter* spp. is one of the most common causative agents of foodborne diseases in Europe (EFSA 2016). It is a zoonotic Gram-negative bacterial pathogen, frequently associated with acute gastroenteritis in both industrialized and developing countries. In most cases, foodborne campylobacteriosis is caused by *Campylobacter jejuni*, however *Campylobacter coli*, and, to a lesser extent, *Campylobacter lari* have also been responsible for occurrence of

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infection. *C. jejuni* is particularly adapted to poultry, which undoubtedly constitutes the largest reservoir of the species. Contaminated water and foods of animal origin, especially improperly processed chicken, have been implicated as vehicles for the transmission of campylobacteriosis.

The clinical symptomatology of illness caused by *C. jejuni* includes diarrhoea, which may range from mild and watery to bloody dysentery. In addition to enteric disorders, *C. jejuni* infection is frequently associated with the development of immunoreactive complications, such as polyarthralgia, Guillain-Barre and Miller-Fisher syndromes, and eventually death (DINGLE et al., 2002).

Unlike other enteric pathogens, such as *Salmonella* spp., Shiga-like toxigenic *Escherichia coli* and Noroviruses, the pathogenesis of *C. jejuni* is poorly understood. It is considered that motility, bacterial adhesion, invasion of the enterocytes and synthesis of toxin and hemolysin seem to be the main virulence factors. So far, it is known that this microorganism, upon initial colonization of the epithelial lining in the jejunum, moves towards the large intestines and eventually settles in the colon, which is the target organ (SILVA et al., 2011; HADDOCK et al., 2010). During primary interaction with enterocytes, *C. jejuni* adheres to them and then is internalized within the cells, causing tissue damage, inflammation and thus gastroenteritis. The factors associated in the invasion are capsular polysaccharide (CPS) and sialylation of the lipooligosaccharides outer core or *Campylobacter* invasive antigens (Cia) (WATSON and GALAN, 2008; LOUWEN et al., 2008). The binding of *C. jejuni* to fibronectin, a component of the extracellular matrix of eukaryotic cells, is mediated by a 37 kDa outer membrane protein (adhesin), termed *CadF,* for *Campylobacter* adhesion to fibronectin. Previous studies have indicated that *C. jejuni* binds to fibronectin on the basolateral surface of human colonic cells (KONKEL et al., 2005). This factor is encoded by the *cadF* (Campylobacter Adhesion to Fibronectin F) gene (ZIPRIN et al., 2001).

C. jejuni possess a single polar flagellum at one or both ends of the cell. The flagellum consists of two proteins, FlaA and FlaB, with significantly more

FlaA incorporated into the flagellar filament (ALM et al., 1993). It is considered that the polar flagellum of *C. jejuni* is the primary adherence factor that establishes contact between the eukaryotic (both human and broiler) cell membrane and specific bacterial-invasion factors, hence facilitating the colonization of the intestinal epithelial cells (HU et al., 2008). The flagellar rotatory motion allows *C. jejuni* to move easily through the viscous mucus layer covering the intestinal epithelial cells, which consequently identifies the flagella as a virulence determinant. This virulence factor is encoded by the *flaA* gene (NUIJTEN et al., 2000).

Additional virulence factors, such as bacterial toxins synthesized by *C. jejuni,* play a role in the development of the disease, inducing enterocolitis. The most important ones include cytolethal distending toxin (CDT) and hemolysin. The *cdtA, cdtB* and *cdtC* genes have been identified for expression of cytotoxin production (PURDY et al., 2000). CDT production is important for interleukin-8 (IL-8) release by intestinal cells, which plays an important role in the host mucosal inflammatory response caused by *C. jejuni* (VAN DEUN et al., 2007). CDT is composed of three subunits: the catalytic subunit CdtB, which is encoded by the *cdtB* gene and has DNase I-like activity, whereas CdtA, and CdtC are binding proteins for delivering CdtB into target cells. Translocation of CdtB to the nucleus induces genotoxic effects on the host DNA, triggering DNA repair cascades that lead to cell cycle arrest and eventual cell death.

Guillain-Barré syndrome (GBS) is an acute, immune-mediated, *post infectionem* illness affecting the peripheral nervous system, and is strongly associated with *C. jejuni* gastrointestinal infection (HUGHES and REES, 1997). Expression of ganglioside-like mimicry in the outer core lipooligosaccharide, and onset of an immune response in the host that cross-reacts with ganglioside-rich targets in the peripheral nerve, are considered to be involved in the pathogenesis of *Campylobacter*-induced GBS (MORAN and PRENDERGAST, 2001). The genes involved in the biosynthesis of ganglioside-mimicking lipooligosaccharide encompass were identified as *cgtB* and *wlaN* (LINTON et al., 2002).

Aim of this study was to estimate the prevalence of *flaA*, *cadF*, *cdtB* and *cgtB* genes associated with virulence in *C. jejuni* isolated from broiler meat originating from different slaughterhouses in Serbia.

Materials and methods

Broiler meat samples. The autonomous province of Vojvodina, and the Western Serbia region were selected as the areas for collection of samples since 69% of the total Serbian broiler production is located in these regions and the surrounding area (RODIĆ et al., 2016). Six large and/or mid-scale slaughterhouses are located in this area. All of them were contacted and asked to collaborate in the study. On the basis of the outcome of this request, sampling was performed in 3 slaughterhouses. Between April and January, a total of 115 broiler meat samples were sampled. Meat samples included parts of carcasses, such as drumsticks (n = 84) and breast fillets (n = 31). All batches sampled from the same slaughterhouse originated from different farms or broilers reared at different periods in the same farm. The broilers were commercially reared and slaughtered at the age of 6 to 7 weeks.

Isolation and identification of Campylobacter. The detection of *Campylobacter* isolates was performed according to the ISO 10272-1:2017 standard, respectively, using microaerophilic conditions generated by the GasPak gas-generating kit (BD, USA). The bacterial isolates were confirmed as *C. jejuni* using their morphological and biochemical features (a positive hippurate hydrolysis test), as well as a multiplex PCR method (a method developed in-house) and Sanger sequencing (described previously by VAN HUNG et al., 2011) of a fragment of the highly conserved *dnaJ* gene encoding DnaJ protein (a member of the Hsp40 family) which co-regulates the active form of heat shock sigma factor 32. Nucleotide sequences of the *dnaJ* gene of the tested strains were deposited in Genbank under accession numbers KJ081720-KJ081742. The strains were cryopreserved and stored at -196 °C until further analysis.

Virulence genes characterization. Cryopreserved bacterial culture was reactivated by streaking on modified charcoal cefoperazone deoxycholate agar (mCCDA) supplemented with CCDA supplement (Oxoid, USA), and incubated for 48 hours under microaerophilic conditions at 42 °C. A loopful of cultivated colonies of each respective strain was resuspended in 200 µL of Prepman Ultra Sample Preparation Reagent (Thermofisher, USA), and vortexed for 10-30 seconds. Samples were placed in a heat block (Eppendorf, Germany) set to 95 °C, for 10 minutes. After cooling, samples were centrifuged in a microcentrifuge (Eppendorf, Germany) at 13.000 $\times g$ for 2 minutes. Nanodrop 1000 (Thermo Scientific, USA) was used to assess the extracted DNA purity, measured as the ratio of absorbance at 260 and 280 nm (A260/280). A 50 µL of the recovered supernatant was used as stock DNA for subsequent PCR reactions, for detection of virulence genes. The reference strain *C. jejuni* ATCC33560 was used as a positive control.

A PCR technique was also used to target the *flaA*, *cadF*, *cdtB*, and *cgtB* genes, respectively. The primer sequences of all the genes, the size of PCR amplicons and the PCR annealing temperature used in this study are shown in Table 1.

All PCR amplifications were performed in a reaction mixture of 50 µL containing 1× AmpliTaq Gold 360 Master Mix (Thermofisher, USA), 0.4 µM of each primer (Microsynth, Switzerland), and 5 µL of the extracted template DNA. Nuclease free water was added to make up the final volume to 50 µL. The reaction mix was subjected to amplification in a PCR thermal cycler (Applied Biosystems, USA). The amplification program for these virulence markers involved initial denaturation at 95 °C for 5 minutes and the cyclic phase was repeated 35 times. Each cycle involved denaturation at 95 °C for 60 s, annealing for 60 s, and elongation at 72 °C for 60 s, with final extension at 72 °C for 10 minutes. The corresponding annealing temperature is shown in Table 1.

Appropriate negative controls, with the PCR master mix, without the DNA template, were included with each PCR run. PCR amplification products were visualized on 1.5% agarose gel (Thermofisher, USA) in Tris-Acetated-EDTA buffer at 100 V. The gel was stained using 1× SYBR Safe DNA Gel Stain (Invitrogen, USA) and 100 bp ladder (Bioline, UK) was used as a molecular-weight size marker.

Table 1. Nucleotide sequences of the primers used, annealing temperatures and the size of the amplicons

Primer name	Sequence	Annealing temperature	Amplicon size (bp)	Specificity	Reference
flaA-f	ATGGGATTTCGTATTAACAC	52 °C	1713	*flaA*	Hanel et al., 2004
flaA-r	CTGTAGTAATCTTAAAACATTTTG				
cadF-f	TTGAAGGTAATTTAGATATG	48 °C	400	*cadF*	Konkel et al., 1999
cadF-r	CTAATCCTAAAGTTGAAAC				
cdtB-f	GTTAAAATCCCCTGCTATCAACCA	55 °C	495	*cdtB*	Pickett et al., 1996
cdtB-r	GTTGGCACTTGGAATTTGCAAGGC				
cgtB-f	TAAGAGCAAGATATGAAGGTG	52 °C	561	*cgtB*	Linton et al, 2002
cgtB-r	GCACATAGAGAACGCTACAA				

Results

Distribution of virulence genes. In total, 115 broiler meat samples were examined for the presence of *Campylobacter* spp. It was found that 89 samples (77.4%) of the broiler carcasses' parts were contaminated with *Campylobacter* spp. Both phenotypical and PCR identification revealed that *C. jejuni* was isolated in 35 samples (39.3%), whereas *C. coli* was detected in the remaining 54 samples (60.7%). All 35 strains of *C. jejuni* were screened for the presence of the aforementioned four virulence genes. The prevalence of the *flaA* gene was 100% (35/35), while the *cadF* gene was detected in 33 isolates (94.3%). Next, the *cdtB* gene was found in 97.1% (34/35) of strains. Finally, the prevalence of the *cgtB* gene was just 5.7% (2/35). The gene prevalence pattern discloses that all strains contained one (*flaA*) virulence gene, while the majority (>94%) of them, besides *flaA*, contained two more virulence markers (*cadF* and *cdtB*). A complete set of four virulence genes was found in just 3 strains.

Discussion

According to EFSA (2017), the occurrence of *Campylobacter* remains high in broiler meat, mostly due to inadequate heat treatment and improper handling. Identification of virulence markers is becoming essential for consumer safety.

In this study, the prevalence of the four genes most frequently involved in the pathogenesis of campylobacteriosis was analysed in a group of *C. jejuni* isolated from broiler carcass parts during the spring-winter period in Serbian slaughterhouses.

The results of our research showed that the *flaA* gene was present in all *C. jejuni* strains tested. Previous studies showed that the detection rate of the *flaA* gene was between 95% (THAKUR et al., 2010; EL-JAKEE et al., 2015) and 100% (WIECZOREK et al., 2012; CASABONNE et al., 2016). Given its high prevalence, it is clear that the *flaA* gene plays a critical role in the bacterial colonization of the ileum and colon, by mean of encoding proteins of the flagellar filament necessary to move against peristaltic movements, penetration of the epithelial mucus, and colonization of enterocytes.

In the group of Serbian *C. jejuni* strains, the prevalence of *cadF* gene was 94.3% *i.e.* this gene was detected in all but 2 strains. In a study by ABU-MADI et al. (2016), they reported s similar prevalence (96.6%), however other researchers have reported that the prevalence of this gene in strains isolated from poultry carcasses, faeces, and from human clinical specimens was 100% (ROZYNEK et al., 2005; TALUKDER et al., 2008; SZCZEPAŃSKA et al., 2015; CASABONNE et al., 2016). The failure to detect the *cadF* gene in 2 strains may be explained by possible mutation(s) in the oligonucleotide sequence meaning that primers are unlikely to anneal the DNA template. It is certain that the *cadF* gene is highly conservative

among the *C. jejuni* species, and the CadF protein was responsible for the initiation of interaction between bacterial cells and host epithelium surface receptors (KONKEL et al., 2005).

In this study, the prevalence of the CDT toxin-encoding gene was investigated. CDT toxin consists of three subunits: CdtA, CdtB, and CdtC. All three proteins are required jointly in order to induce the cytotoxic effect. However, it is considered that only the CdtB subunit has enzymatic activity, and mediates DNA degradation and cell cycle blocking (PURDY et al., 2000). PCR testing of the *C. jejuni* strains in our study revealed that almost all of them harboured the *cdtB* gene. (34/35). This was in accordance with reports published by THAKUR et al. (2010); GONZALEZ-HEIN et al. (2014); SZCZEPAŃSKA et al. (2015); CASABONNE et al. (2016). Although it is generally accepted that the *cdtB* genes are widespread amongst poultry, cattle and human isolates in different countries (VAN DEUN et al., 2007), there have been some contradictory results when it comes to the prevalence of *cdtB* in Asia. Namely, RIZAL et al., 2010 reported an unusual low prevalence of *cdt*A, *cdtB* and *cdt*C genes in chicken meat (13.33%, 20%, 35%, respectively) in India. The same authors speculated that the results occurred due to genetic reasons or variations in the isolates from different geographic areas. However, TALUKDER et al. (2008) reported that in Bangladesh the prevalence of *cdtA* was 97.5%, *cdtB* 97.5%) and *cdtC* 97.5%.

Finally, the prevalence of the *cgtB* gene in *C. jejuni* strains from Serbia was quite low - 5.7%. This gene is closely associated with the onset of Guillain-Barre syndrome since it encodes lipooligosaccharide that cross-reacts with ganglioside-rich targets in the peripheral nerve. Data on the frequency of *cgtB* are scarce; so far KORDINAS et al. (2005) has reported that the prevalence in human *C. jejuni* strains was 24.4%, NGUYEN et al. (2016) found prevalence of 55.6%, while CASABONNE et al. (2016) detected the *cgtB* gene in just 6.7% of strains.

Conclusion

In summary, the high prevalence of the three genes in *C. jejuni* associated with motility, intestinal colonization and toxin synthesis confirmed

that these pathogenic markers are widespread among the isolates from broiler meat in Serbian slaughterhouses. Furthermore, in this study, a high genetic heterogeneity was found in the *C. jejuni* population, showing a pattern partially different from other reported virulence genes. Combining the prevalence of this genes with the fact that the infective dose is low (500 cfu/g) indicates that the consumption of undercooked broiler meat may pose a serious threat to veterinary public health and consumers.

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SAŽETAK

Campylobacter jejuni vodeći je uzročnik crijevnih bolesti koje se očituju proljevom kod ljudi. Cilj ovoga istraživanja bio je utvrditi prevalenciju različitih gena virulentnosti u izolatima dobivenima iz mesa brojlera u klaonicama u Srbiji. Na prisutnost vrsta roda *Campylobacter* spp. ukupno je pretraženo 115 uzoraka. Morfološko-biokemijskim pretragama, multipleks PCR tehnikom kao i sekvenciranjem visokoočuvane regije gena *dnaJ*, koji kodira sintezu proteina DnaJ iz obitelji proteina Hsp40, 35 izolata identificirano je kao *C. jejuni*. Izolati su pretraženi na prisutnost četiri gena patogenosti: *flaA, cadF, cdtB* i *cgtB*. Ti su geni odgovorni za ekspresiju adherencije, kolonizacije, sinteze citotoksina *C. jejuni* kao i nastanak Guillain-Barreova sindroma. Izolati su pokazali široku varijaciju u prisutnosti tih gena. Svi su izolati bili pozitivni na gen *flaA*, pri čemu je u populaciji *C. jejuni* iz ovoga istraživanja utvrđena visoka heterogenost u genetskoj strukturi, koja je djelomice različita od rezultata drugih autora. Kod 94,3 % izolata *C. jejuni* utvrđen je gen *cadF*, kod 97,1 % gen *cdtB*, a kod 5,7 % gen *cgtB*. Ovo istraživanje pruža početne podatke o prevalenciji i distribuciji gena *flaA, cadF, cdtB* i *cgtB* kod *C. jejuni* izoliranih iz mesa brojlera u klaonicama u Srbiji.

Ključne riječi: *Campylobacter jejuni,* virulentnost, geni, brojleri