Prevalence of Virulence Genes in Strains of *Campylobacter jejuni* Isolated from Broiler Products, Children, Wild Birds and Dairy Cattle in Lithuania

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Keywords: *Campylobacter jejuni, virulence genes, prevalence.*

Abstract. *The present study was designed to investigate the prevalence of virulence genes in C. jejuni from different sources. A total of 98 C. jejuni strains from different sources were selected for the study: 34 strains isolated from broiler products (B), of which 17 from fresh poultry and 17 from marinated poultry products; 17 strains from children clinical samples (V); 31 strains from wild birds' faeces (LK), of which 16 from pigeons and 15 from crows; and 16 from dairy cattle faeces (G). Nine virulence genes (cadF, virB11, ceuE, gltA, hcp, cdtA, cdtB, cdtC, flaA) were selected for the study. The identification of the virulence gene was performed by polymerase chain reaction, the visualization of DNA amplicons was done by electrophoresis, and photos were taken in ultraviolet light.*

The study revealed that the virB11 gene was found only in 6% of C. jejuni strains isolated from dairy cows, while this gene was not detected in Campylobacter strains isolated from other sources. The gltA gene was found in all tested C. jejuni strains. The cdtA virulence gene was prevalent in examined Campylobacter strains as only 3% of wild bird strains lack this gene. The CdtC gene was detected in all C. jejuni strains isolated from the children clinical samples, wild birds and chicken, whereas campylobacters isolated from the cattle did not harbour this gene (19% of all tested strains). The study results revealed that occurrence of virulence genes was differently distributed among strains of C. jejuni and occurrence of certain virulence genes (hcp, cdtC, flaA) depended on the origin of strain isolation.

Introduction

Campylobacter spp. cause a foodborne intestinal infectious disease called campylobacteriosis. The infection is spread by faecal-oral route. It can also be transferred through insufficiently heat-treated meat, especially poultry and poultry products, and uncooked or unpasteurized milk contaminated with these bacteria. Water can also be a source of infection as well as contact with infected animals (ULAC, 2018). The main source of these bacteria is considered to be poultry (Wysok and Wojtacka, 2018). However, dairy cattle, pigs and wild birds are also potential sources of infection for humans. Campylobacteriosis was the most common gastrointestinal bacterial disease in humans in the European Union in 2019, and this tendency has been observed since 2005 (EFSA, 2021). The incidences of campylobacteriosis in 2019 (43.8/100 000) increased by 32.7% compared with 2018 (33.0/100 000) in Lithuania (ULAC, 2019). *Campylobacter spp*. is characterized by a wide variety of virulence factors. Virulence factors include bacterial mobility, chemotaxis, invasion, adhesion, toxin production, bile resistance, drug resistance, and other. Virulence is also related to the genetic diversity of bacteria. Virulence genes such as *cdtA*, *cdtB*, *cdtC* are involved in the production of bacterial toxins, *virB11* shows the extent of invasion in host cells, and *ceuE* encodes proteins (Reddy and Zishiri, 2018). In order to obtain more information on the ability of *Campylobacter* to cause gastrointestinal disease in humans, it is important to assess the prevalence of virulence genes in *Campylobacter* strains isolated from different sources. A kind of or similar study has not been performed in Lithuania before; therefore, with this study we aim to evaluate and compare the prevalence of virulence genes in *C. jejuni* strains isolated from different sources like the dairy cattle, wild birds, broiler products and children clinical samples.

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Materials and methods

Bacterial strains

A total of 98 *C. jejuni* strains from different sources were selected for the study: 34 broiler products (B), of which 17 fresh poultry and 17 marinated poultry products; 17 children (V); 31 wild birds (LK), of which 16 were pigeons and 15 crows; and 16 dairy cattle samples (G). All isolates were from the Campylobacter collection at the Department of Food Safety and Quality, Veterinary Academy, Lithuanian University of Health Sciences. The identification of Campylobacter isolates was performed with multiplex PCR as described by Wang et al. (2002) with the minor modifications described previously by Ramonaite et al. (2015).

DNA isolation

Campylobacter isolates were stored as frozen stocks at –80°C in brain heart infusion broth (BHI) (Oxoid Ltd., Basingstoke, UK) with 30% glycerol (Stanlab, Poland). They were recovered from frozen stocks on blood agar base No. 2 (Oxoid, Basingstoke, Hampshire, England) supplemented with 5% defibrinated horse blood (E&O Laboratories, Burnhouse, Bonnybridge, Scotland) and incubated under microaerophilic conditions (5% oxygen, 10% carbon dioxide and 85% nitrogen) at 37°C for 48 h.

One 10 µL loop of bacterial culture grown on blood agar plates was collected and suspended in 200 µL of PrepMan Ultra (Applied Biosystems, Foster City, USA). The suspension was vortexed for 15–30 s in order to dissolve the bacterial culture and subsequently heated at 100°C for 10 min. Afterwards, the samples were centrifuged at 16 000 g for 3 min. The supernatant containing bacterial DNA was used immediately or transferred to a new tube and stored at –20°C until use.

PCR detection of virulence determinants

Amplifications of the nine virulence genes (*FlaA*, *CadF*, *VirB11*, *CeuE*, *CdtA*,*CdtB*, *CdtC*, *GltA*, *Hcp*) were performed in separate tubes. Final volume of 25 µL PCR reaction mix was composed of 7.25 µL Dream-TaqGreen PCR Master Mix (Thermo Scientific, Waltham, USA), 15.75 µL Milli-Q water, and 1 µL of primers mix. Finally, a 24 µL mix was prepared in separate tubes, adding 1 µL of chromosomal DNA. The samples were centrifuged at 4000 g for 1 min. DNA amplification was carried out in a thermocycler using different programs. For *FlaA*, *CadF*, *VirB11* genes, an initial denaturation step was performed at 94°C for 5 min, 95°C for 1 min followed by 30 cycles of amplification, 1 min for specific temperature (*FlaA* – 53°C, *cadF* – 5°C, *virB11* – 53°C), extension at 71°C for 1 min and ending with final extension at 72°C for 5 min (Wysok and Wojtacka, 2018). For *CeuE* gene, an initial denaturation step was at 95ºC for 3 min, 95°C for 30 s followed by 30 cycles of amplification, 57°C for 30 s, extension at 72°C for 1 min and ending with final extension at 72°C for 5 min (Gonzalez et al., 1997). For *CdtA, cdtB, cdtC* genes, an initial denaturation step was at 94°C for 1 min, 94°C for 1 min followed by 30 cycles of amplification, 42°C for 2 min, extension at 72°C for 3 min and ending with final extension at 72°C for 5 min (Pickett et al., 1996). For *Hcp* and *gltA* genes, an initial denaturation step was at 94°C for 5 min, 95°C for 1 min followed by 30 cycles of amplification, 60°C for 1 min, extension at 71°C for 1 min and ending with final extension at 72°C for 5 min (Harrison et al., 2014). Each PCR product (11 mL) was loaded into a 2% TopVision LM GQ agarose gel (Thermo Scientific) containing 6.5 µL ethidium bromide and analysed by gel electrophoresis. The PCR products were visualized on a UV board. The GeneRuler 100 bp DNA Ladder (Thermo Scientific) was used as the molecular size marker (Fig. 1).

Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2007 and IBM SPSS Statistics 24 software packages. The Crosstabs procedure was used to assess the dependence in between the samples of quantitative data. Chi^2 was also calculated. In addition, descriptive statistics was applied to get quantitative variables data. A P value of < 0.05 was used to indicate statistically significant results.

Results

Overall 98 *C. jejuni* strains from 4 different sources were selected for examination: 34 strains isolated from broiler products, 17 isolated from children clinical samples, 31 strains from wild birds and 16 from dairy cattle faeces. The study revealed that three virulence genes were detected in all strains despite the source of isolation. The study also showed that none of examined *C. jejuni* strains harboured all nine or eight virulence genes (Table 1). Whereas five virulence genes were detected in all *C. jejuni* strains isolated from broiler and wild bird faeces samples.

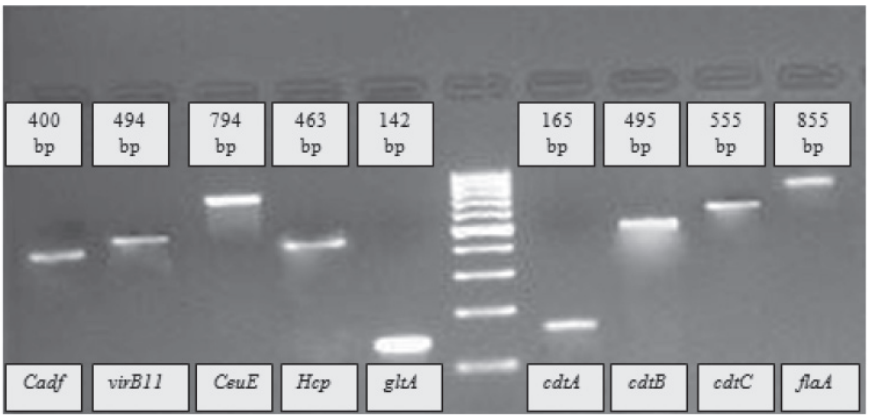

Fig. 1. Visualization of amplified virulence genes, gene fragment lengths 100–1000 (bp)

All examined strains contained from 1 to 3 virulence genes. However, several strains harboured up to 6 or 7 virulence genes (Table 1).

We identified only one *Campylobacter* strain isolated from a cattle faeces sample (6%) harbouring the *VirB11* gene, which is responsible for *Campylobacter spp.* invasion and the distribution among different sources. The *gltA* virulence gene was found in all strains isolated from different sources (100%). The *FlaA* virulence gene, responsible for invasion and mobility, was not found in *Campylobacter* strains isolated from the broiler and children clinical samples. However, this gene was detected in the wild bird (61%) and in the cattle (63%) strains. The *Hcp* virulence gene, contained in the type VI secretory system (T6SS) responsible for the cellular transfer of proteins, was mainly found in *C. jejuni* strains isolated from the broiler product strains (21%), but not found at all in bovine Campylobacter strains. The *CadF* virulence gene, which promotes bacterial attachment to intestinal epithelial cells, was found in all *Campylobacter* strains isolated from the wild bird and children clinical samples (100%) with lower frequency of detection in bovine strains (94%). The *CeuE* gene, encoding lipoprotein, was mostly found in *Campylobacter* strains isolated from the children clinical samples (71%) and rarely detected in cattle strains (19%). The *CdtA* virulence gene responsible for toxin production was not found in only one strain of the wild birds whereas it was prevalent in all strains isolated from other sources. The *CdtB* gene related with toxin producing was found in all *Campylobacter* strains isolated from the broiler and wild bird samples. In *Campylobacter* strains isolated from the cattle and children clinical samples, this gene was found in 94% of the strains. The *CdtC* virulence gene associated with production of toxins was not found in only three *Campylobacter* strains isolated from the cattle (prevalence 81%). The prevalence was 100% in *Campylobacter* strains isolated from broilers, wild birds, and children clinical samples (Table 2).

Discussion

This study evaluated the prevalence of 9 major virulence genes in *C. jejuni* strains isolated from 4 different sources such as broiler products, dairy cattle, wild birds, and children clinical samples. Our study revealed that the distribution of the *virB11* virulence gene responsible for *Campylobacter* invasion among different sources was very low and detected in only one Campylobacter strain isolated from the cattle. Other studies have revealed that the *virB11* gene is rarely found in campylobacters isolated from cattle. In a study conducted in 2003, the *virB11* virulence gene was detected in only one of the 15 *Campylobacter* strains isolated from cattle (Bang Det al., 2003). However, in a study conducted in Poland in 2018, the examination of 99 *Campylobacter* strains (50 pigs and 49 cattle samples) revealed that this gene prevailed in 50% of the strains (Wysok and Wojtacka, 2018). After determining the distribution of the virulence gene *cadF* – which promotes bacterial attachment to intestinal epithelial cells – among different sources of *Campylobacter*, it was found that the prevalence of *Campylobacter* strains isolated from children and wild birds was 100%. Only in one *Campylobacter* strain out of 16 strains isolated from cattle, the *cadF* virulence gene was not detected. A similar study by Danish and Iranian researchers showed that the *cadF* virulence gene was detected in all *C. jejuni* strains isolated from

Table 1. Quantity of virulence genes in Campylobacter strains depending on the source of isolation

Source	Number of strains	Quantity of virulence genes (number of strains / percentage)								
		1	2	3	4	5	6	7	8	9
Broilers	34	34/100	134/100	134/100	134/100	134/100	21/62	7/21	0/0	0/0
Children	17	117/100	117/100	117/100	117/100	915/94	12/71	3/18	0/0	0/0
Wild birds	31	131/100	131/100	131/100	131/100	131/100	18/61	7/23	0/0	0/0
Cattle	16	116/100	116/100	116/100	915/94	814/88	6/38	2/13	0/0	0/0
	98	198/100	198/100	198/100	97/99	94/96	56/58	18/19	0/0	0/0

Table 2. Prevalence of virulence genes in *Campylobacter* strains depending on the source of isolation

Source	Number of strains	Identified virulence genes (number of tested strains / percentage of identified genes in strains)								
		cadF	*virB11*	*Hcp*	*gltA*	*ceuE*	*cdtA*	*cdtB*	*cdtC*	*flaA*
Broilers	34	33/97	0/0	7/21	34/100	22/65	34/100	34/100	34/100	0/0
Children	17	17/100	0/0	3/18	17 /100	12/71	17/100	16/94	17/100	0/0
Wild birds	31	31/100	0/0	1/3	31/100	14/45	30/97	31/100	31/100	12/61
Cattle	16	15/94	1/6	0/0	16/100	3/19	16/100	15/94	13/81	6/63
	98	96/98	1/1	11/11	98/100	51/52	97/99	96/98	95/97	18/18

cattle (15 strains) and children (200 strains) (Bang et al., 2003; Ghorbanalizadgan et al., 2014). The study of the *hcp* virulence gene prevalence among *Campylobacter* strains isolated from different sources revealed that the gene was mostly common among *C. jejuni* strains isolated from cattle (100%) and wild birds (97%). The *hcp* virulence gene is in the type VI secretory system (T6SS) responsible for protein delivery to a "target" cell. The prevalence of this gene in broiler products was 21% (7 strains of 34). Similar results were obtained in a 2015 study in the United Kingdom, where the prevalence of the virulence gene in poultry was 28.8% (detected in 17 poultry strains of 59 tested) (Corcionivosch et al., 2015). In the study of the *gltA* gene distribution among different sources, it was found that the *gltA* gene was 100% common in all of them. A study by other researchers showed that the *gltA* virulence gene was detected in all *C. jejuni* tested strains (59 strains) isolated from poultry (Corcionivoschi et al., 2015). In a study of the prevalence of the *ceuE* virulence gene – responsible for encoding lipoprotein – among *Campylobacter* strains isolated from different sources, it was revealed that this virulence gene was the least common among *C. jejuni* strains isolated from cattle and wild birds (19% and 45%, respectively). A similar study by other researchers showed that the *ceuE* virulence gene was detected in 14 of 15 *Campylobacter* strains that were isolated from cattle (Bang et al., 2003). To determine the prevalence of the virulence gene *cdtA* – responsible for toxin production – among *Campylobacter* strains isolated from different sources, the prevalence among *C. jejuni* strains isolated from broilers, children, and cattle was found to be 100%. In a study conducted in Denmark, the prevalence of the *cdtA* gene in *C. jejuni* strains isolated from cattle was 100% (15 strains) (Bang et al., 2003). And in a study conducted by scientists in 2018, the prevalence of this virulence gene in poultry samples was 96% (152 strains tested) and 100% in *C. jejuni* strains isolated from humans (155 strains) (Wieczorek et al., 2018). The *cdtB* virulence gene – responsible for toxin production – was detected in all *C. jejuni* strains isolated from broilers and wild birds in the study of the *cdtB* virulence gene prevalence. In a similar study, the prevalence of this gene in poultry samples was 94.1% (152 strains studied) (Wieczorek et al., 2018). A study on the prevalence of *cdtC* virulence gene – also responsible for toxin production – among *Campylobacter* strains isolated from different sources revealed that this gene was detected in 100% of *C. jejuni* strains isolated from broilers, children and wild birds. In a similar study conducted by researchers in 2018, it was found that the *cdtC* virulence gene was detected in 97.4% of *Campylobacter* strains isolated from birds (152 tested) and in 100% of *Campylobacter* strains isolated from humans (155 strains) (Wieczorek et al., 2018). In a study of the *flaA* virulence gene prevalence – responsible for mobility and invasion – among *Campylobacter* strains isolated from different sources, it was found that this gene was not detected in *C. jejuni* strains isolated from broilers and children. The prevalence among wild birds was 39%, and in the cattle samples, it was 38%. In a similar study, the *flaA* virulence gene was detected in 100% (15 strains) of *C. jejuni* strains isolated from cattle (Bang et al., 2003). In a study conducted in 2018, the prevalence of this gene in *C. jejuni* strains isolated from poultry reached 98.7% (152 tested) (Wieczorek et al., 2018).

Our study revealed that *Campylobacter jejuni strains* isolated from different sources are distinct in relation to the diversity of genes encoding virulence. The knowledge about the prevalence of virulence genes, depending on the isolation source of *campylobacters*, is important in assessing potential threats to consumer health, as these genes encode the ability of bacteria to cause the disease and the severity of the caused disease.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

This research has received funding from the European Regional Development Fund (project No. 09.3.3.-LMT-K-712-09-0066) under agreement with the Research Council of Lithuania (LMTLT).

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Received 6 April 2021
Accepted 23 June 2021