RESEARCH ARTICLE

Virulence comparison of human and poultry *Campylobacter jejuni* isolates in a mouse model

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ABSTRACT

Objective: Research into *Campylobacter jejuni* pathogenesis and host responses to *C. jejuni* infection is needed in the fight against human campylobacteriosis.

Methods: We established intravenous infections of BALB/c mice with either a *C. jejuni* food isolate or *C. jejuni* of human origin. Further we include PCR to demonstrate the presence and stability of the putative virulence genes *cadF, virbB11, cdtB, cdtC, ceuE* in *C. jejuni* isolates and we examined cytokine production of IL-6, IL-12, TNF-α, IFN-γ, IL-10 in the livers of these infected mice.

Results: We confirm here the presence of the *cadF, cdtB, cdtC* and *ceuE* genes in a food and a clinical *C. jejuni* isolate, with no sequence changes after the *C. jejuni* sub-culturing in a food model and when recovered from mouse liver after infection. Both of these *C. jejuni* isolates persisted in the mouse livers and activated comparable cytokine patterns for IL-12, TNF-α, IFN-γ and IL-10, with down-regulation of IL-6.

Conclusions: These data show the comparability of these *C. jejuni* food and clinical isolates in terms of the prevalence and stability of their putative virulence genes and the outcome of disease during systemic murine campylobacteriosis.

Highlights

- We investigated virulence of *C. jejuni* food and clinical isolates in BALB/c mice
- The *cadF, cdtB, cdtC* and *ceuE* genes are important for the pathogenesis of infection
- *C. jejuni* activated comparable local cytokine production in livers of infected mice

Introduction

Campylobacter jejuni is a common bacterium that causes acute bacterial gastroenteritis/ enterocolitis in humans in developed countrie[1]. Consumption of undercooked poultry products contaminated with campylobacters and cross-contamination in the kitchen environment during food preparation have been identified as risk factors for human campylobacteriosis[2]. Genetic diversity of campylobacters and the possibility of their adaptation to changing environments might represent critical mechanisms for their survival outside the host and during host–pathogen interactions.

Although the entire genome of *C. jejuni* has been sequenced, the mechanisms of pathogenesis and host responses to *C. jejuni* infection are still poorly understood[3]. Histological analyses of biopsy samples from patients with campylobacter colitis have shown that after adhesion in the gut, these bacteria can traverse the epithelial barrier to enter the underlying tissue, and to eventually disseminate throughout the body[4].

During infection, both pro-inflammatory and anti-inflammatory cytokines are induced, which is important in the disease pathology and for the determination of whether the immune system is successful in providing protection against specific pathogens[5,6]. Human monocytes/ macrophages are considered as the early host responders to infection, and when the body is infected with *Campylobacter* spp., these cells produce a range of cytokines and chemokines, including interleukin (IL)-1α, IL-1β, IL-6, IL-8, and tumour necrosis factor (TNF)-α. It has been previously reported that lipopolysaccharide from *C. jejuni* induces the production of the pro-inflammatory IL-1 and TNF-α[7].

We previously introduced an *in vivo* experimental model of systemic murine campylobacteriosis to demonstrate *C. jejuni* dissemination and tissue invasion[8]. In the present study, we established intravenous infections of BALB/c mice with either a *C. jejuni* food isolate or *C. jejuni* of human origin. We compared the presence and stability of the putative virulence genes (*i.e.*, *cadF, virbB11, cdtB, cdtC, ceuE*) and the virulence properties of both of these *C. jejuni* isolates, as well as cytokine production (*i.e.*, IL-6, IL-12, TNF-α, IFN-γ, IL-10) in the livers of these infected mice.

Materials and Methods

Campylobacter Isolates

Campylobacter jejuni K49/4 food isolate from poultry meat (Laboratory for Food Microbiology, Biotechnical Faculty, University of Ljubljana, Slovenia) and *C. jejuni* 705080 clinical isolate (Laboratory for Diagnostics of Enteric Infections, Teaching Institute of Public Health of the County Primorsko Goranska, Croatia) were cultured microaerobically (5% O_2, 10% CO_2, 85% N_2) in Preston broth (Oxoid, CM0689) at 42 °C.

Virulence Genes and Sequence Analysis

Five genes were assessed using polymerase chain reaction (PCR) and sequence analysis: the *cadF* gene that encodes adhesin, which is responsible for certain steps in cell invasion[9]; the *virB11* gene that is a marker for the pVir plasmid[10]; the *cdtB* and *cdtC* genes that are involved in the production of cytolethal distending toxin[11]; and the *ceuE* gene that encodes a lipoprotein that is associated with haemolysins[12]. The bacterial DNA was extracted using the PrepMan Ultra sample preparation reagent (Life Technologies, 4318930), following the manufacturer recommendations, and the extracted DNA preparations were stored at -20 °C. PCR amplifications were performed in 50 μL reaction volumes that contained 10× RED Taq

PCR buffer (Sigma-Aldrich GmbH, B5926), 20 mM dNTP (Life Technologies, N8080007), 300 nM forward primer, 300 nM reverse primer (Table 1), 1 U/µL RED Taq polymerase (Sigma-Aldrich GmbH, D4309), and 5 µL DNA lysate. The PCR was performed in a thermal cycler PCR system (2400 GeneAmp; Perkin Elmer). The primer sequences of the virulence genes, the expected sizes of their products, and the cycling conditions varied according to the reference for the specific gene determination. The PCR products were electrophoresed on 2% agarose gels. The virulence genes were further investigated by sequence analysis (Macrogen), to study the genetic differences of these *C. jejuni* food and clinical isolates following storage conditions with sub-cultivation in chicken juice agar as a food model[13] and also after infection of BALB/c mice, for *C. jejuni* recovered from the mouse liver on day 3 post-infection. Briefly, for the preparation of chicken meat juice, commercially frozen chickens without giblets were placed in a container, unwrapped and thawed at room temperature. Afterwards the collected chicken meat juice was centrifuged (10,000 rpm for 10 min) and before sterilization (through a 0.45 µm filter) we added 2 % Agar Bios Special LL (Biolife, 4110302). The sequences of the genes have been deposited with GenBank, with the accession numbers given in Table 1.

Table 1. Target genes, PCR primer pairs and GenBank accession numbers of the *C. jejuni* food and clinical isolates

Target [reference]	Primer pair	Sequence (5'-3')	Amplicon size (bp)	*C. jejuni* food isolate			*C. jejuni* clinical isolate		
				Food model[a]	Mouse liver[b]	GenBank accession No.	Food model[a]	Mouse liver[b]	GenBank accession No.
cadF ([10])	F2B	TGG AGG GTA ATT TAG ATA TG	400	X	X	KJ875964	X	X	KJ875965
	R1B	CTA ATA CCT AAA GTT GAA AC							
virB11 ([11])	virB11	GAA CAG GAA GTG GAA AAA CTA GC	708	ND	ND	/	ND	ND	/
	virBR	TTC CGC ATT GGG CTA TAT G							
cdtB ([12])	VAT2	GTT AAA ATC CCC TGC TAT CAA CCA	495	X	X	KJ875955	X	X	KJ875956
	WMI-R	GTT GGC ACT TGG AAT TTG CAA GGC							
cdtC ([12])	WMI-F	TGG ATG ATA GCA GGG GAT TTT AAC	555	X	X	KJ875958	X	X	KJ875959
	LPF-X	GTT GGC ACT TGG AAT TTG CAA GGC							
ceuE ([13])	JEJ1	CCT GCT CGG TGA AAG TTT TG	794	X	X	KJ875962	X	X	KJ875963
	JEJ2	GAT CTT TTT GTT TTG TGC TGC							

[a], Isolate from -80 °C and after sub-culturing in a food model;

[b], Isolate from the mouse liver

X, gene present; ND, gene not detected

Campylobacter Infection in Mice

Eight- to 12-week-old BALB/c (H-2d) mice were obtained from the Central Animal Facility of the Faculty of Medicine, University of Rijeka. The experiments were conducted according to the Guidelines of the International Guiding Principles for Biomedical Research Involving Animals[14]. The Ethical Committee of the University of Rijeka approved all of the animal experiments described here. The mice were infected intravenously via the lateral tail vein with a single dose (200 μL) of 0.5-1.0 $\times 10^9$ CFU *C. jejuni* cells. The mice were sacrificed at different times from their infection, and their livers were removed and immediately frozen in liquid nitrogen and stored at −80 °C until required for assay. The *C. jejuni* CFU were determined for the livers as previously described[15]. For cytokine analyses, two sets of mice were infected with each of the *C. jejuni* isolates (*i.e.*, food isolate, clinical isolate), with at least three mice per group.

Cytokine Analysis

The cytokine concentrations in the livers of control and infected mice were measured in duplicate as previously described[16], using mouse cytokine enzyme-linked immunosorbent assay kits (Thermo Scientific), and the data are expressed in pg/mL liver homogenate. The assay sensitivity levels were: IL-6, 7 pg/mL; IL-12, 5 pg/mL; TNF-α, 9 pg/mL; IFN-γ, 10 pg/mL; IL-10, 12 pg/mL. The control mice were of the same age and sex, and were injected with sterile saline. All of the experiments were independently repeated three times.

Statistical Analysis

As the data were not normally distributed, they were expressed as the median and the range for each group of mice. The Mann-Whitney U test was used for nonparametric comparison of the median cytokine values. The differences were considered significant at a *P* level of 0.05. The statistical analysis was performed using SPSS 15.0 for Windows (Statsoft Inc.).

Results

Presence of Virulence Factors in C. jejuni

For both of the tested *C. jejuni* food and clinical isolates, the presence of the virulence genes *cadF*, *cdtB, cdtC* and *ceuE* was confirmed as indicated with symbol X in Table 1, along with the absence of *virB11* as indicated with symbol ND for not determined, using PCR (Table 1). Furthermore, the DNA sequences of these genes were compared for each *C. jejuni* isolate obtained after 48 h cultivation on Columbia selective agar with additional sub-cultivation in the model food medium in a microaerophilic atmosphere at 42 °C, and in the isolates from the livers of the infected BALB/c mice. We confirmed the stability of each of the virulence genes present (*i.e.*, *cadF, cdtB, cdtC, ceuE)* in both the *C. jejuni* food and clinical isolates (Table 1).

Cytokine Profile in Systemic Murine Campylobacter Infection

We established systemic infection of BALB/c mice with intravenous inoculation of 0.5-1.0 $\times 10^9$ CFU *C. jejuni* from the food and clinical isolates. Three to five mice were infected per experimental group. We observed the mice 8 days post-infection. The mice showed signs of illness, including ruffled fur, a hunched posture, and weight loss, but none of them died. At different times (*i.e.*, days 1, 3 and 8 post-infection), the mice were euthanized, their livers were removed aseptically and homogenised, and their *C. jejuni* CFU were determined. These data showed that campylobacters were present in the liver during the whole experimental period. On day 1 post-infection, there were 7 log CFU/liver for the food isolate and 6 log CFU/liver for the

clinical isolate, with a 1 log reduction after 8 days. These small differences in CFU/liver between the food and clinical isolates were not significant (data not shown).

As the successful clearance of bacteria depends on the appropriate response of the host immune system, potential differences in the induction of cytokine production were determined in the livers during these infections with the campylobacter strains of different origins. The livers from parallel sets of three to five mice per time point were used to determine the concentrations of the selected cytokines (*i.e.*, IL-6, IL-10, IL-12, TNF-α, IFN-γ) in the organ homogenates (Figures. 1, 2). The differences in the liver concentrations of the cytokines detected in the uninfected control mice and in the *C. jejuni*-infected mice were generally significant, although the differences in the cytokine induction between the two investigated *C. jejuni* strains were significant only for IFN-γ (on the first day post-infection), and TNF-α and IL-10 (both on the eighth day post-infection). All of the investigated cytokines were produced in markedly higher amounts in the infected mice, compared to the uninfected control mice, with the exception of IL-6, the production of which was down-regulated (Figure 1). It can also be seen that the *C. jejuni* food isolate induced the highest cytokine production at the beginning of the infection (day 1), and that the cytokine concentrations in the livers of the mice infected with the *C. jejuni* clinical isolate showed an ascending trend towards the last experimental day. Figure 2 shows the ratios between the selected pro-inflammatory cytokines and IL-10 in the livers of the infected mice. These pro-inflammatory cytokine/IL-10 ratios showed no significant differences between the tested *C. jejuni* strains.

Figure 1

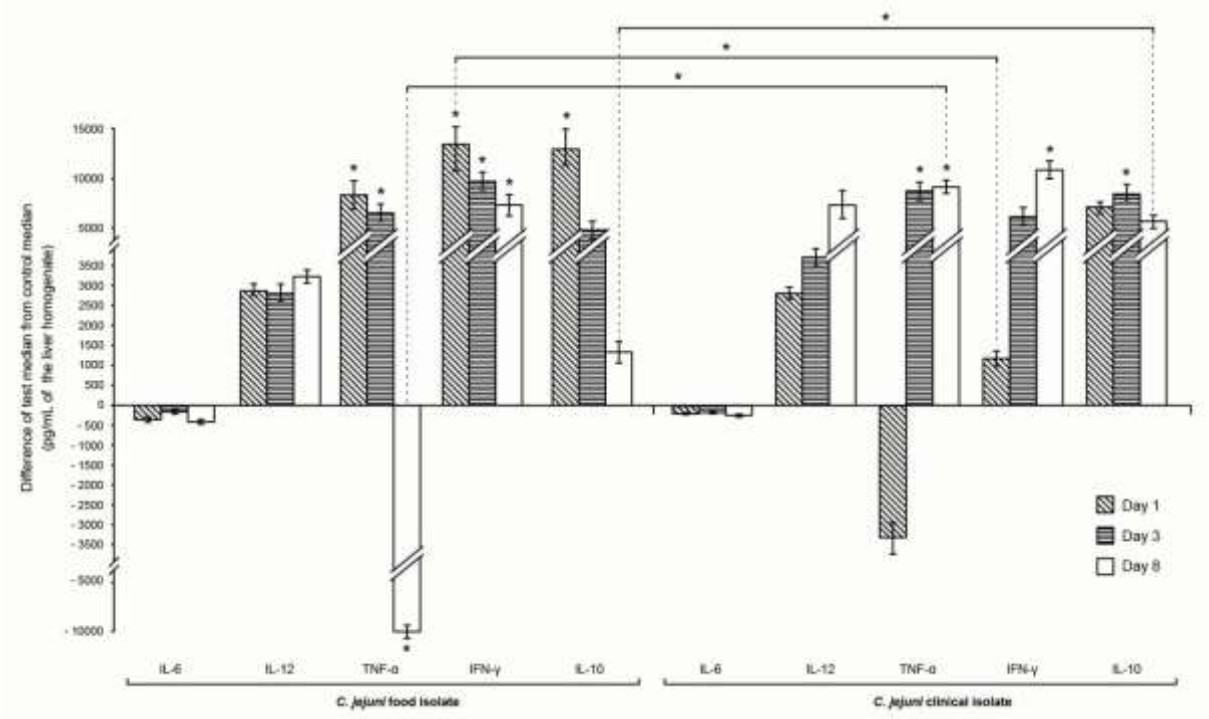
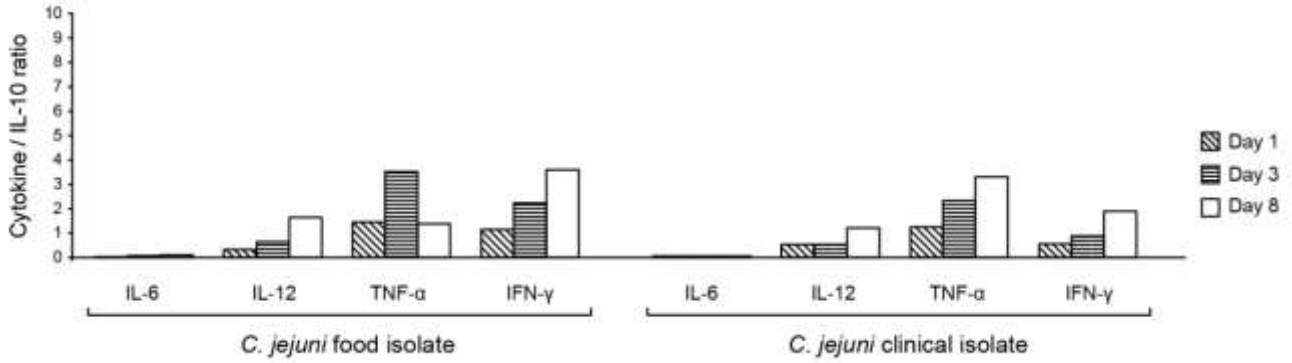


Figure 2



Discussion

Campylobacteriosis is the leading human bacterial food-borne illness and the most frequently reported zoonosis throughout the world, with *C. jejuni* responsible for around 90% of reported cases. According to reports of official monitoring systems, human infections are mostly transmitted via natural cycles from environmental waters, animals and the food chain[17]. Stress responses of *C. jejuni* to treatments used during food processing and the modulation of *C. jejuni* virulence represent potential problems in the safety of the food production chain. We previously investigated the influence of several stress factors (*i.e.*, heat, starvation, oxygen) on campylobacter virulence properties *in vivo*, where we noted that *C. jejuni* adaptation during a stress challenge was crucial not just for pathogen survival out of the host, but also during host–pathogen interactions, and thus for bacterial pathogenicity[15,16].

On the basis that campylobacters are very variable genetically, in the present study, we investigated whether potential differences in the virulence genes between a food and a clinical *C. jejuni* isolate can influence their ability to colonise and induce cytokine production *in vivo*. First, for both the *C. jejuni* food and clinical isolates, using PCR, we determined the presence of the virulence genes that encode potential virulence factors: *cadF*, which encodes an outer-membrane protein that facilitates the binding of campylobacter in the host[9]; *cdtB* along with *cdtC* and *cdtA*, which encode cytolethal distending toxin[18]; and *ceuE*, which encodes a lipoprotein component of a binding-protein-dependent transport system for the siderophore heterochelin included in iron acquisition[1]. Previous studies have indicated the high prevalence of these genes in different *C. jejuni* isolates and their importance for *C. jejuni* virulence[20]. Also using PCR, in both the *C. jejuni* food and clinical isolates, we confirmed the absence of virulence gene *virB11*, which encodes an invasion-linked marker that is associated with bacterial adherence and invasion[21]. This is similar to previous studies that have shown variations for the presence of *virB11* in comparable prevalence studies of *C. jejuni* isolates from poultry, pig and cattle meat, and from human isolates, with *virB11* present in <20% of these isolates[20]. Hence, the absence of the *virB11* gene indicates that both of the *C. jejuni* isolates tested in the present study cannot produce the pVir plasmid. It has been suggested that this system modulates the *C. jejuni* virulence, as isogenic virB11 mutants demonstrated reduced *in vitro* adherence and invasion in the INT 407 cell line, and reduced in-vivo virulence in a ferret diarrhoeal disease model, compared to the wild type[10].

We followed the stability of the virulence genes in these *C. jejuni* food and clinical isolates,

with their detection by PCR and sequencing in isolates following sub-culturing in chicken juice agar under storage conditions at -80 °C, and also in isolates from the livers of the infected mice. Sequence analysis of both of the tested *C. jejuni* isolates showed that the sequence data of the virulence genes *cadF, cdtB, cdtC* and *ceuE* remained the same after their sub-culturing in chicken juice agar as a model of long-term presence of *C. jejuni* in the food chain, and after transmission through the mouse host and their collection from the liver of the infected mice on day 3 post-infection.

The prevalence of these virulence genes in both of the tested isolates and their stability through the imitation of the transmission route using their long-term presence in chicken juice and their transmission through a host model, provide information relating to the potential transmission of campylobacters from food to the human host. Thus, we further monitored the possible differences in *C. jejuni* colonisation and cytokine induction in the murine model of infection. In previous studies, we showed that after intraperitoneal injection *C. jejuni* can produce systemic infection *in vivo* what was confirmed by the isolation of the *C. jejuni* from the livers and spleens of infected mice[8] and successfully invade epithelial cells *in vitro*[21]. Our earlier *in vivo* data[22] are in agreement with the conclusions of Day et al.[23], who considered *C. jejuni* to be a facultative intracellular pathogen, and not a typical extracellular bacterium. In the present study, we focussed on the *C. jejuni* clearance from the livers of BALB/c mice infected with these *C. jejuni* food and clinical isolates, and the local cytokine responses following *C. jejuni* dissemination.

Hu et al. [7] demonstrated that *C. jejuni* infection triggers innate inflammatory responses, and induces human dendritic cell maturation and production of pro-inflammatory cytokines, including IL-6, IL-8, IL-12 and TNF-α. In this way, these cytokines can induce *Campylobacter*-specific Th1 effector-cell responses and contribute to the pathogenesis and clinical symptoms of campylobacter infection. We expected similar findings during murine infection, with noticeable differences between the bacterial strains used for infection. All of the cytokines persisted well during the whole period of infection. The cytokine profiles varied between the different strains of *C. jejuni* not so much in terms of the quantities detected, but more in terms of the dynamics of their production. Infection with both of these *C. jejuni* strains induced strong pro-inflammatory responses with elevated levels of IL-12, TNF-α and IFN-γ, and the opposite for IL-6. Indeed, IL-6 can also act as an anti-inflammatory cytokine[24]. The most pronounced differences were for TNF-α and IL-10 production, which decreased towards the end of the experimental period when the *C. jejuni* food isolate was used for infection of the mice.

Previous studies have demonstrated central roles for IFN-γ, IL-1 and TNF-α in inflammatory reactions that can lead to eradication of obligate and facultative intracellular pathogens[25]. TNF-α has been shown to have a role in limiting the severity of bacterial infections by various mechanisms, including selective killing of cells harbouring bacteria, activation of monocytes and granulocytes, and stimulation of specific immune responses. Particularly in infections with facultative intracellular bacteria, anti-TNF-α antibody treatment promotes massive proliferation of the infecting organisms. These data suggest that the ability to inhibit TNF-α would be advantageous to the pathogen[26]. Higher numbers of bacteria isolated from the livers of mice infected with the *C. jejuni* food isolate in the present study confirm these observations of Beuscher et al. [26].

In conclusion, campylobacters are widely known for their rapid adaptive ability and genomic instability[27], as they have a wide range of strategies to colonise and invade the human host, despite the presence of multiple host defence mechanisms[28]. Thus, it is important to enhance the understanding of the host response mechanisms. In the present study, we report on the presence of the virulence genes *cadF*, *cdtB*, *cdtC* and *ceuE* and their genetic stability in both *C. jejuni* food and clinical isolates. Local cytokine production in the livers of mice infected with these bacteria showed no marked differences between these two *C. jejuni* strains, which confirm the roles of these genes in the pathogenesis of infection. It will be important to further investigate the likeliness of damage of these putative virulence genes or their products during food processing, which in the case of the consumption of contaminated foodstuff of poultry origin, would not result in human infection.

Conflicts of Interest

There are no conflicts of interest.

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