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Research Paper

Prevalence of virulence genes in strains of *Campylobacter jejuni* isolated from human, bovine and broiler

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Abstract

Campylobacter jejuni isolates of different origins (bovine, broiler meat, human) were screened by polymerase chain reaction for the presence of 4 genes *cdt*B, *cst-II*, *ggt*, and *vir*B11, previously linked to virulence such as adherence, invasion, colonization, molecular mimicry, and cytotoxin production. In addition, the isolates were screened for the presence of the global gene regulator *csr*A linked to oxidative stress responses, biofilms formation, and cell adhesion. All the *C. jejuni* isolates were positive for *cdtB* gene. The *csr*A gene was detected in 100% and 92% of *C. jejuni* isolates from human and animal origin and the *virB11* gene was detected in 7.3% and 3.6% isolates from chicken and human respectively. All isolates from bovine were negative for the *vir*B11 gene. The isolates showed a wide variation for the presence of the remaining genes. Of the *C. jejuni* recovered from human 83.6%, and 32.7% were positive for *cst-II*, and *ggt* respectively. Out of the isolates from chicken 40% and 5.5% isolates revealed the presence of *cst-II*, and *ggt*, respectively. Finally of the *C. jejuni* isolates from bovine, 97.7% and 22.7% were positive for *cst-II*, and *ggt* respectively. We conclude that the genes of this study circulate among humans and animals. These results led us to hypothesize that the isolates associated with enteritis (*cdt*B positives) are not selected by environmental or host-specific factors. On the other hand, the high frequencies of *csrA* gene in *C. jejuni* show that this gene is important for the survival of *C. jejuni* in animals and humans.

Key words: *Campylobacter jejuni*, virulence, Guillain Barré syndrome, broiler, bovine.

Introduction

Campylobacter spp. is the leading cause of bacterial foodborne enteric disease in Europe (European Union, European Food Safety Authority and European Centre for Disease Prevention and Control, 2012) while in the USA, *Campylobacter* spp. has been ranked among the most important bacterial foodborne pathogens (Scallan *et al.*, 2011). Clinical syndromes vary from mild to severe and from enterocolitis to extraintestinal diseases such as the Guillain-Barré Syndrome (GBS) (Islam *et al.*, 2009). Fifty to 80% of human infection may be attributed to the chicken (European Union, European Food Safety Authority, 2010), but also cattle-related cases (via undercooked beef or unpasteurized milk and dairy products) have been reported (Sheppard *et al.*, 2009). A study in Chile showed that 38-68% of broiler meat after chilling is contaminated with *C. jejuni* (Figueroa *et al.*, 2009). Studies from our laboratory, recently detected genetically indistinguishable isolates between broiler meat and human campylobacteriosis in Chile by pulsed-field gel electrophoresis (PFGE) (González-Hein *et al.*, 2013). In 2010, Chileans on average consumed 33.3kg of poultry meat being the main source of animal protein (Chile, Office of Agricultural Policies, Trades and Information, 2012). All this emphasizes the importance of chickens as a potential reservoir and source of *C. jejuni* infection in Chile.

The natural heterogeneity of *C. jejuni* has made studying the pathogenicity of this pathogen particularly

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challenging (Croinín and Backert, 2012). However, in recent years, significant progress has been made to increase our understanding of the role of several key factors associated with bacterial virulence mechanisms such as the cytolethal distending toxin (CDT) (Ge *et al.*, 2008) as well as the molecular mimicry process in GBS (Koga *et al.*, 2006; Louwen *et al.*, 2008).

Cell invasion of epithelial cells and CDT production are important bacterial virulence mechanisms that induce enterocolitis. Cell invasion could result in cellular injury, leading to reduced absorptive capacity of the intestine, whereas CDT production is important for interleukin-8 (IL-8) release by intestinal cells *in vitro* which plays an important role in the host mucosal inflammatory response caused by *C. jejuni* (Hickey *et al.*, 2000; Deun *et al.*, 2007). CDT is composed of three subunits: the catalytic subunit CdtB, which is encoded by the *cdt*B 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 host DNA, triggering DNA repair cascades that lead to cell cycle arrest and eventual cell death. In addition it has also been suggested that CDT may play a role in adhesion and invasion (Konkel *et al.*, 2001; Jain *et al.*, 2008).

C. jejuni is also the major cause of the GBS, a post infectious autoimmune -mediated neuropathy (Koga *et al.*, 2006). The development of this autoimmune neuropathy after *C. jejuni* infection is thought to be primarily related to sialylated lipooligosaccharides (LOS) on the cell surface of *C. jejuni* that exhibit molecular mimicry with gangliosides on peripheral nerves (Nachamkin *et al.*, 2002). First Van Belkum *et al.* (2001) showed that a sialyltransferase encoded by the *cst*-II gene in *C. jejuni* is associated with risk of developing GBS, and later studies have confirmed this link (Nachamkin *et al.*, 2002; Koga *et al.*, 2006). On the other hand, the cst-II gene has been linked to the invasiveness of *C. jejuni* for intestinal epithelial cells (Louwen *et al.*, 2008).

The *C. jejuni* gene *ggt* encoding the periplasmic gamma-glutamyltranspeptidase (GGT) seems to play a pivotal role in the enteric colonization. GGT has been shown in chicken model to be important in long lasting gut colonization, and *in vitro* it has been shown that GGT plays a significant role in *C. jejuni*-mediated apoptosis (Barnes *et al.*, 2007). This genetic determinant has also been proposed as a host associated genetic marker (Gonzalez *et al.*, 2009), hence we were interested in assessing the presence of *ggt* and in investigating the possible association with *C. jejuni* strains from broiler origin.

Another virulence gene linked with *Campylobacter* spp. invasiveness is the invasion-associated marker (*vir*B11) gene. In vitro studies have shown that this genetic marker of *C. jejuni* strains is associated preferentially with both adherence and invasion (Bacon *et al.*, 2000).

Although many genes related to the pathogenicity of *C. jejuni* have been reported, the relationships between these genes and the sources of strains are not clear.

C. jejuni is ubiquitous in the aerobic environment and possess regulatory systems to sense and adapt to external stimuli, such as oxidative and aerobic (O2) stress (Gundogdu *et al.*, 2011). Considering the limited contingent of regulatory effectors found in *C. jejuni* genomes, it has been suspected that the gene encoding the regulatory protein CsrA might play a vital role in the regulation of stress responses and virulence determinants in this pathogen. It was demonstrated that the global posttranscriptional regulator *csrA* (*c*arbon *s*tarvation *r*egulator) favors biofilm formation, adherence of intestinal epithelial cells and survival to oxidative stress, suggesting an important regulatory role for this gene in *C. jejuni* pathogenesis (Fields and Thompson, 2008).

In this paper, we assess the presence of a set of genes associated with virulence in *C. jejuni* isolates of different sources (broiler meat, bovine and human) to determine whether host-specific or environmental factors select for or against a set of genes related to virulence in *C. jejuni* isolates.

Material and Methods

Bacterial isolates

The *C. jejuni* isolates (n = 154) were obtained from the strain collection at the Microbiology and Probiotics Laboratory of the Food Technology and Nutrition Institute, University of Chile and the Microbiology Laboratory of the Pontificial Catholic University of Chile. All 154 *C. jejuni* isolates were collected in the Metropolitan Region during 2006 to 2010. Among the isolates, 55 were from stool specimens of diarrheal patients (sporadic cases), the remaining 55 strains were obtained from chicken carcasses and 44 were obtained from bovine rectal swabs. The confirmation of the samples was carried out by standard microbiological methods. The hippurate hydrolysis test was used for determination of the *C. jejuni* strains. All hippurate-positive isolates were determined as *C. jejuni*.

PCR of genes associated with virulence

DNA from isolates was extracted by standard molecular biological techniques using the kit: Genomics DNA Purification (Bioingentech, Concepción, Chile). The DNA from all isolates was amplified by PCR as a control for DNA extraction and *C. jejuni* species confirmation by analysis of the 16SrRNA (cccj gene). Then all isolates were screened by polymerase chain reaction (PCR). Amplification of the *cdtB, csrA, cst-II, ggt* and *virB11*, locus were carried out in a master mix volume of 15 µL containing buffer 1X (5X Green GoTaq® Flexi Buffer Promega, Madison, WI, U.S), 0.4 mM each dNTP's (Promega, Madison, Wisconsin, United States (U.S)), 0.06 U/µL GoTaq® Flexi

DNA polymerase (Promega, Madison, Wisconsin, U.S), 2.4 mM Magnesium Chloride Solution(Promega, Madison, Wisconsin, U.S) and 0.7 μM each of forward primer and reverse primer (IDT®, Coralville, Iowa, U.S)). Amplification of the *ggt* gen varied in the concentrations of $MgCl_2$ (2.0 mM) and primers (0.5 μM). Primers and PCR conditions generated in this study are given in Table 1. Amplified products were visualized in agarose gels (1.5%) stained with ethidium bromide. DNA of *C. jejuni* 81176 and Staphylococcus aureus ATCC® 25923 were used as positive and negative control respectively. In addition, a reaction control was also included (Mix with IDT® water used as template). The reference strain *C. jejuni* 81176 was isolated during an outbreak of *Campylobacter* diarrhea associated with raw milk consumption in Minnesota and it has been widely used in pathogenesis studies (Korlath *et al.*, 1985).

Sequencing of PCR products

Five PCR products of *C. jejuni* 81176 were purified using the Kit Wizard® SV gel and PCR clean-up system, (Promega, Madison, Wisconsin, U.S) (Figure 1). Finally, the PCR products were automatically sequenced in both directions at Pontifical Catholic University of Chile. Sequencing was done on an ABI PRISM® 3130 Applied Biosystems. For bioinformatics analysis of the sequences and alignments, Blast and ClustalW2 software were used and matched with the database. The sequences of each gene were shown to be rather conserved (95 to 100% similarity within each gene).

Statistical analyses

The Chi-square test or Fisher's exact test when necessary were used to test for similarity in the frequencies of

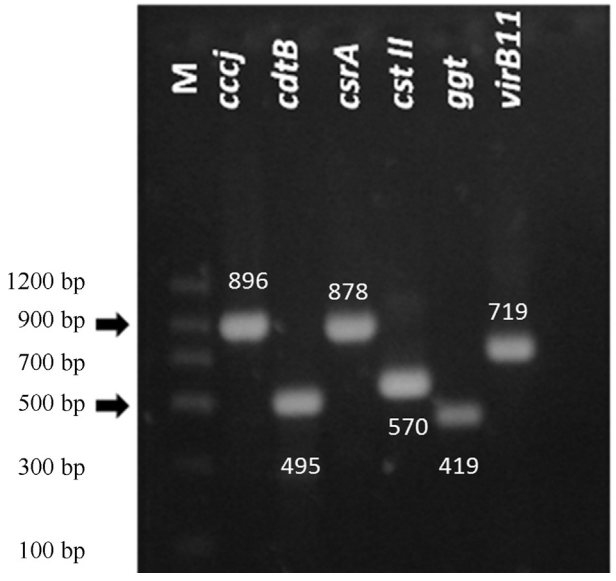

Figure 1 - Purified polymerase chain reaction products from the *cccj*, *cdtB*, *csrA, cst-II, ggt* and *virB11* genes. Strain: *C. jejuni 81176.* M, DNA Molecular Weight Marker.

Table 1 - Primers and conditions used in the fragments amplification of the five genes associated with virulence.

Gen target and PCR conditions	Primers	Sequence (5'-3')	Amplicon
16S ribosomal gene de *Campylobacter coli* and *C.jejuni*, 94 °C for 3 m/ 4 cycles 94 °C for 30 s, 54 °C x 30 s and 72 °C x 35 s /30 cycles of 94 °C x 30 s, 56 °C x 30 s y 72 °C x 35 s, /5 min x 72 °C/ 65 °C for 5 min	ccjFW ccjRV	GCG TAG GCG GAT TAT CAA GT ATT CCA CTG TGG ACG GTA AC	896 pb
*cdt*B gene, 94 °C for 3 min/ 4 cycles of 94 °C for 30 s, 54 °C x 30 s y 72 °C x 35 s /30 cycles de 94 °C x 30 s, 56 °C for 30 s and 72 °C x 35 s, /5 min a 72 °C/ 65 °C for 5 min	cdtBFW cdtBRV	CAC GGT TAA AAT CCC CTG CT GCA CTT GGA ATT TGC AAG GC	495 pb
*vir*B11 gene, 94 °C for 3 min/ 3 cycles of 94 °C for 30 s, 55 °C for 30 s y 72 °C for 30 s/ 35 cycles of 94 °C for 35 s, 57 °C for 35 s and 72 °C for 35 s/ 10 min for 72 °C/ 65 °C for 5 min	Vir FW Vir RV	GGT GGA ACA GGA AGT GGA AA AGG TTA TTT CCG CAT TGG GC	719 pb
*csr*A gene, 94 por 3 min/ 30 cycles of 94 °C x 30 s, 58 °C for 30 s and 72 °C for 30 s / 5 min for 72 °C/ 65 °C for 5 min	csrAFW csrARV	CAC AGT CAG TGA AGG TGC TT ACT CGC ACA ATC GCT ACT TC	878 pb
ggt gene, 94 °C for 3 min/ 30 cycles of 94 °C x 30 s, 58 °C for 30 s and 72 °C for 30 s / 5 min for 72 °C/ 65 °C for 5 min	ggtFW ggt RV	GAG TGC TAT GCT TGA TCG CT TAG GTG GCG ACA TGG AAA TG	419 pb
*cst*II gene, 94 °C for 3 min/5 cycles of 94 °C by 35 s, 52 °C for 35 s and 72 for 35 s and 72 °C x 35 s/ 5 min for 72 °C /65 °C for 5 min	cstIIFW cstIIRV	CAG CTT TCT ATT GCC CTT GC ACA CAT ATA GAC CCC TGA GG	570 pb

genes within the isolates from different hosts, using alpha level of 0.05.

Results

Distribution of virulence genes

In each of the 154 isolates, the *16Sr*RNA gene was detected by PCR. Thus, all isolates could be confirmed as *C. jejuni*. Additionally, this PCR was suitable to control the DNA extraction procedure. The *cdt*B gene was also present in 100% of *C. jejuni* isolates tested, regardless of their origin. Similarly the regulator gene *csr*A was identified as an habitual virulence gene in the *C. jejuni* isolates. The frequency of detection of *csr*A, *cst*-II, and *ggt* genes varied between human, bovine and chicken isolates. From a total of 154 isolates, 146 (94.8%), 111 (72%) and 32 (20.8%) tested positive for *csrA*, *cst*-II, *ggt* respectively being the three genes more frequently detected in bovine and in diarrheagenic human isolates. Finally, results also indicated that only 3.9% (6 of 154) of the *C. jejuni* strains were *vir*B11- positive and this gene was not detected in the bovine strains. The identification of these genes by PCR is depicted in Table 2. This figure shows the distribution of these genes associated with virulence according to the source of the isolates.

Statistical analysis of the distribution of the five genes among the various origins of the isolates: in this analysis, *csr*A, *cst*-II and *ggt* genes had significantly different frequencies ($p < 0.05$) for isolates from different sources of origin using the Chi-square test or Fisher's exact test.

In human isolates *csr*A gene was found with higher frequency than in chicken isolates ($p < 0.05$). In bovine and human *C. jejuni* isolates *cst*-II gene was found with higher frequency than in chicken isolates ($p < 0.05$). On the other hand, a lower amount of isolates from chicken meat harbored the *ggt* gene as detected by PCR. Besides, the frequency of bovine and human strains harboring *ggt* gene was higher than chicken meat isolates ($p < 0.05$). The distribution between the isolates of different origins harboring genes *cdtB,* and *virB11* was similar in all group tested ($p > 0.05$).

Discussion

The majority of *C. jejuni* research has been focused using European isolates or from the U.S. origin. The occurrence of virulence and toxin genes among *C. jejuni* isolates from different sources has been studied poorly in South America. As far as we know there are some reports in Brazil, where a set of virulence-associated genes were detected in a substantial proportion of children with diarrhoea (Quetz *et al.*, 2012) and less in chicken (Carvalho *et al.*, 2010).

In this study all the strains investigated harbored *cdt*B gene. It is indeed generally accepted that the *cdt*B genes are widespread amongst poultry, cattle and human isolates in Denmark, Japan, Poland, and Belgium (Bang *et al.*, 2003; Datta *et al.*, 2003; Rozynek *et al.*, 2005; Deun *et al.*, 2007). However, low percentages of occurrence of *cdt*B have been reported in humans (28%) and chickens (20%) in India, which could be due to genetic reasons or variation in the isolates from different geographic areas (Rizal *et al.*, 2010).

There are other human infection sources of campylobacteriosis beyond chicken and cattle. However, the equal distribution of *cdtB* in all human infectious sources of campylobacteriosis, as in chicken, and cattle, and the crucial role of CDT in the intestinal pathology - persistence of infection in the gastrointestinal tract and in the severity of mucosal inflammation (Ge *et al.*, 2008; Jain *et al.*, 2008) - led us to suggest that no selection for or against CDT associated strains of *C. jejuni* occurs in these animals. Georgiades and Raoult (2011) hypothesized that the only truly identifiable phenomena, witnessing the convergent evolution of the most pathogenic bacteria for humans are the loss of metabolic activities, *i.e.*, the outcome of the loss of regulatory and transcription factors and the presence of protein toxins.

To date, only three studies have included the detection of GBS-related genes for human and animal isolates (Parker *et al.*, 2005; Hardy *et al.*, 2011; Amon *et al.*, 2012). Parker *et al.* (2005) detected genes responsible for ganglioside mimics in 64% of the human enteric and animal isolates. Recently in Austria, Amon *et al.* (2012) also frequently detected the *cst*-II gene in isolates of bovine, poultry and human. Our analysis of the *cst*-II gene detection indicates the presence of potentially risky *C. jejuni* strains from bovine feces and contaminated broiler meats. It is not clear why the detection of *cst*-II from all sources is so high, although these findings seem to suggest that these ganglioside-mimicking LOS structures are advantageous to *C. jejuni* colonization of various hosts. Louwen *et al.* (2008) demonstrated that the disruption of *cst-II* significantly af-

Table 2 - Presence of virulence-associated genes in *Campylobacter jejuni* strains isolated from chicken carcasses, bovine and human, Chile.

Origin	Number of isolates	*cdt*B	*csr*A	*cst*-II	*ggt*	*vir*B11
Human	55	55(100%)	55(100%)	46(83.6%)	18(32.7%)	2(3.6%)
Broiler meat	55	55(100%)	48(87.3%)	22(40%)	3(5.5%)	4(7.3%)
Bovine	44	44(100%)	43(97.7%)	43(97.7%)	10(22.7%)	0(0%)
Total	154	154(100%)	146(94.8%)	111(72%)	32(20.8%)	6 (3.9%)

fects the invasiveness of *C. jejuni* for intestinal epithelial cells. What is clear is that the production of ganglioside-mimicking LOS structures alone is not sufficient to elicit GBS; other bacterial and/or other types of factors as the individual immune system are also required (Amon *et al.*, 2012). The data generated revealed that the gene encoding the regulatory protein CsrA is present in all examined diarrheagenic human strains of *C. jejuni*, and is highly conserved among animals. A similar situation has been shown by Barnard *et al.* (2004) who detected the regulator *csra*A gene in all examined clinical strains of *Helicobacter pylori*. In this pathogen, a close relative of *C. jejuni,* CsrA is involved in the regulation of several virulence phenotypes, including motility, oxidative stress resistance, and mouse colonization (Barnard *et al.*, 2004).The high frequency of *csrA* gene in *C. jejuni* allows us to hypothesize that this regulator is important for the survival of *C. jejuni* in the broiler meat, bovine and human. Recently a novel *C. jejuni* transcriptional regulator, Cj1556 that is involved in oxidative and aerobic stress responses, ability to form biofilms, and survival of *C. jejuni* was identified (Gundogdu *et al.*, 2011).

The *ggt* gene has been recognized more frequently in human and chicken isolates (Gonzalez *et al.*, 2009). Our findings diverged from those reported by Gonzalez *et al.* (2009), who suggested the *ggt* gene to be chicken associated based on PCR analysis of *C. jejuni* strains isolated from humans, chickens, and cattle. This may reflect differences between the *C. jejuni* populations in livestock in Chile and those in Finland; however, our results stress the need to confirm the results obtained using a set of *C. jejuni* strains from diverse geographical origins. Nevertheless, it is interesting to note that *ggt*-positive strains predominated in human isolates (33%) as it has been reported by other authors in Europe (Barnes *et al.*, 2007; Gonzalez *et al.*, 2009; Zautner *et al.*, 2011). This is consistent with the hypothesis that *C. jejuni* isolates with an extended amino acid metabolism are more prevalent in humans (Gonzalez *et al.*, 2009). So the acquisition of a gene encoding a gamma-glutamyltranspeptidase enabled this strain to utilize glutamine and glutathione and enhanced its ability to colonize the intestine (Hofreuter *et al.*, 2008). Although we have found a low frequency of *ggt* in broiler isolates, it is necessary to stress that it is clear that successful colonization of chicken GI tract is a multifactorial process in which several genes involved in all areas of the colonization process of *C. jejuni* play a role (Hermans *et al.*, 2011).

The *vir*B11 gene was present equally in the broiler meat strains as in the human strains, suggesting that, at least, some broiler isolates potentially could invade the human intestine. It should be noted, however, that this gene is present in a very small subset of *C. jejuni* isolates (Bacon *et al.*, 2000) and in this study was not detected in isolates from bovine feces.

The overlap observed in distribution of the *C. jejuni* genes among human, bovine, and chicken isolates (Table 2), together with the high consumption of meat by the Chilean population, suggests that human campylobacteriosis may be linked with chicken and bovine meats or unpasteurized milk.

The prevalence of *C. jejuni* virulence genes and their relationship with clinical severity in humans and the expression of virulence factors should be further investigated. It is known that source tracking depends on accurate estimation of the frequency of different genes in each host reservoir. The frequencies in which *ggt* gene were detected in *C. jejuni* isolates from cattle and broiler meat in the present study reveal that the potential use of this molecular genetic marker associated to determined hosts (European Union, European Food Safety Authority, 2010; Zautner *et al.*, 2008) is controversial. The use of additional genotypic methods such as PFGE and/or Multilocus sequence typing that provides more discriminatory power is strongly recommended.

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